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NUCLEIC ACIDS IN THE KIDNEY IN CLOUDY SWELLING.*

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Received for publication February 5, 1953.

IT is known that in cloudy swelling the nuclei of cells stain very faintly with haematoxylin as compared with the normal. Such an appearance has been constantly observed, but no adequate explanation has yet been provided. We have suspected that a correlation may exist between this pale staining of the swollen nuclei and their nucleic acid content. It was therefore considered worth while to investigate, by histochemical and chemical methods, whether any such changes occurred in the nucleic acid content of renal cells affected by cloudy swelling; the results are described in the present paper.

MATERIALS AND METHODS.

Adult Wistar rats (strain WAG-2A) and guinea-pigs, bred in the institute, were used. Cloudy swelling of the kidney was produced experimentally by methods previously employed (Fonnesu, 1952, 1953; Fonnesu and Severi, 1952). In one set of experiments rats were injected intraperitoneally with the smallest dose of *S. typhi murium* toxin which would kill such animals in 4 days. The animals were killed by a blow on the head 24 hr. after injection and were then decapitated and allowed to bleed freely. The kidneys were quickly excised and samples taken for chemical analysis and histological examination. In another set of experiments rats were given 1 mg. of $HgCl_2$/150 g. body wt. subcutaneously, these animals also being killed 24 hr. after the injection. In the third set of experiments the kidney damage was induced by unilateral ligation of the renal pedicle (performed under ether anaesthesia) and the rats were killed 1½ hr. later. The guinea-pigs were injected subcutaneously with diphtheria toxin (1 M.L.D./250 g. body wt.) and killed by decapitation 24 hr. later. Groups of rats and guinea-pigs, litter mates of the experimental animals, were used as controls.

Histological and histochemical methods.

Sections were stained with haematoxylin and eosin to ascertain the type of lesion produced.

Deoxyribonucleic (DNA) and ribonucleic (RNA) acids were stained by Cowdry's modification of the Feulgen reaction (1928) and by Brachet's method (1940) respectively.

Chemical analyses.

For determination of water content the kidneys were weighed, dried in an oven at 100° to constant weight, and then re-weighed. The dry weight was taken as the weight of the solids in the sample.

* The present research has been supported by a grant from the "Consiglio Nazionale delle Ricerche-Roma."

Deoxyribonucleic (DNA) and ribonucleic (RNA) acids were extracted with hot trichloroacetic acid and were estimated colorimetrically (Schneider, 1945).

Statistical analysis.

Symbols and terminology used in the present paper are those given in modern statistical text-books (*cf.* Fisher, 1950; Barbensi, 1952).

RESULTS.

Histology.

Sections stained with haematoxylin and eosin.

Sections stained with haematoxylin and eosin showed that the renal changes induced in rats by *S. typhi murium* toxin were very similar to those produced in guinea-pigs by diphtheria toxin. In both cases the cells of the cortical tubules were swollen, often occluding the whole lumen, and the enlarged nuclei were paler than normal.

In rats that received $HgCl_2$ the kidney damage was more severe; necrosis was present in some zones and several tubular casts were seen in the cortex and in the medulla. In those cortical tubules which did not appear necrotic the cells were swollen and their nuclei stained less intensely than normally.

In the kidneys injured by renal pedicle ligation haemorrhagic foci and cells undergoing necrosis were observed in the sections, but the swelling of the tubular cells and nuclear pallor were less evident than in the toxin-treated animals.

Sections stained for DNA.

In sections prepared from the kidneys of rats injected with *S. typhi murium* toxin and stained by the Feulgen reaction (Fig. 2) the nuclei in the proximal convoluted tubules, besides being markedly swollen, stained much paler than the controls (Fig. 1). The renal cortical tubules of guinea-pigs treated with diphtheria toxin showed similar changes (*cf.* Fig. 5 and 6). On the other hand, in the kidneys damaged by $HgCl_2$ (Fig. 3) or by anoxia after renal pedicle ligation (Fig. 4), apart from the necrotic areas, the cell nuclei were only slightly swollen and the Feulgen reaction only slightly less intense than in the control sections.

Sections stained for RNA.

No changes could be seen histochemically in the RNA content of the kidneys of animals injected with bacterial toxins. In rats poisoned with $HgCl_2$ and in those submitted to renal pedicle ligation, a marked fall in the RNA content was observed only in the necrotic areas, while in the areas not affected by necrosis the stain for RNA was the same as in the control sections.

Chemical Investigations.

The data given in Table I show that the water content of the kidneys in parenchymatous degeneration, produced by various means, did not differ significantly from that of the controls ($P > 0{\cdot}05$).

In Table II the results of DNA and RNA determinations in the kidneys of the control and experimental rats are shown. The DNA content of the kidneys

showing cloudy swelling, produced by either *S. typhi murium* toxin, by $HgCl_2$ poisoning, or by the ligation of the renal pedicle, was significantly less than that of the controls. A statistically significant decrease in the RNA content was observed only after $HgCl_2$ poisoning or after the ligation of the renal pedicle.

In guinea-pigs injected with diphtheria toxin the findings were similar to those observed in rats injected with *S. typhi murium* toxin; *i.e.*, while there was a significant decrease only in the DNA content of the kidneys, although the decrease was not as great as in the rats (*cf.* Tables II and III).

TABLE I.—*Water Content of Kidneys in Cloudy Swelling with Controls (g./kg. of Fresh Whole Kidney).*

Experiment Number.	Rat.				Guinea-pig.	
	Controls.	*S. typhi murium* toxin.	$HgCl_2$.	Pedicle ligation.	Controls.	Diphtheria toxin.
1	769	780	721	801	776	778
2	759	756	731	767	767,	792
3	752	752	734	768	777	793
4	763	776	786	762	767	786
5	743	766	746	750	764	789
6	767	765	780	737	770	764
7	763	762	800	766	751	785
8	759	775	760	751	792	770
9	763	770	773	760	770	744
10	749	746	755	749	784	791
Mean±	759±	765±	759±	761±	772±	779±
Standard error	2·62	3·48	8·23	5·43	3·57	4·97
Difference between the means	—	6	0	2	—	7
t	—	1·379	—	0·331	—	1·144
P	—	>0·05	—	>0·05	—	>0·05

TABLE II.—*Nucleic Acid Content of Rat Kidneys in Cloudy Swelling Compared with Controls.*

Experiment Number.	DNA phosphorus*†				RNA phosphorus*†			
	Controls.	*S. typhi murium* toxin.	$HgCl_2$.	Pedicle ligation.	Controls.	*S. typhi murium* toxin.	$HgCl_2$.	Pedicle ligation.
1	33·0	25·5	27·0	33·0	26·0	19·0	20·0	23·5
2	45·0	26·5	37·5	43·5	35·5	31·5	24·5	29·0
3	39·0	25·5	24·0	21·0	30·0	27·5	31·5	18·0
4	50·0	29·0	23·0	25·0	30·0	24·5	18·5	29·0
5	48·0	39·0	24·5	31·5	34·0	28·5	20·0	24·0
6	50·0	41·0	36·0	32·0	31·5	30·5	23·5	28·5
7	48·0	40·0	39·0	42·0	27·5	31·0	21·5	29·0
8	45·0	28·5	24·0	44·5	25·5	21·5	19·0	24·5
9	47·5	42·5	35·5	46·0	31·0	33·5	18·5	27·5
10	40·0	31·0	19·0	31·0	32·5	32·5	17·0	27·5
Mean±	44·5±	32·8±	28·9±	34·9±	30·3±	28·0±	21·4±	26·0±
Standard error	1·76	2·20	2·29	2·73	1·04	1·54	1·34	1·13
Difference between the means	—	11·7	15·6	9·6	—	2·3	8·9	4·3
t	—	4·164	5·597	2·954	—	1·237	5·235	2·810
P	—	<0·05	<0·05	<0·05	—	>0·05	<0·05	<0·05

* μg. per 100 mg. of fresh whole kidney.
† Phosphorus calculated by pentose determinations (Schneider, 1945).

DISCUSSION.

The results (Table I) show that there is no significant change in the water content of the kidneys in cloudy swelling. The values obtained for the normal and diphtheria toxin-treated guinea-pigs are similar to those found in rabbits by Popják (1948). This worker, using a technique slightly different from ours, obtained results that referred to the fat-free tissue. Our figures were obtained without fat extraction.

The changes observed chemically and histochemically in the nucleic acid content of the kidney of rats injected with *S. typhi murium* toxin were very similar to those found in guinea-pigs treated with diphtheria toxin (Table III).

TABLE III.—*Nucleic Acid Content of Guinea-pig Kidneys in Cloudy Swelling Compared with Controls.*

Experiment number.	DNA phosphorus*†		RNA phosphorus*†	
	Controls.	Diphtheria toxin.	Controls.	Diphtheria toxin.
1	42·0	28·5	32·5	30·5
2	50·5	45·0	34·0	26·5
3	46·5	37·5	30·5	27·0
4	42·0	36·0	31·0	29·0
5	43·5	35·5	34·5	31·0
6	48·0	40·0	33·5	31·5
7	51·5	43·0	38·0	33·5
8	48·0	45·0	27·5	27·5
9	53·5	46·5	27·0	25·5
10	51·5	40·5	31·5	33·0
Mean±	47·7±	39·8±	32·0±	29·5±
Standard error	1·31	1·75	1·04	0·91
Difference between the means	—	7·9	—	2·5
t	—	3·607	—	1·812
P	—	<0·05	—	>0·05

* μg. per 100 mg. of fresh whole kidney.
† Phosphorus calculated by pentose determinations (Schneider, 1945).

In both cases a significant decrease in the DNA content was found by chemical determinations; this fall was parallelled by the pallor of nuclei in histological sections stained by the Feulgen reaction.

EXPLANATION OF PLATES.

FIG. 1.—Normal proximal convoluted tubules of rat kidney. Feulgen reaction for DNA. (× 520).

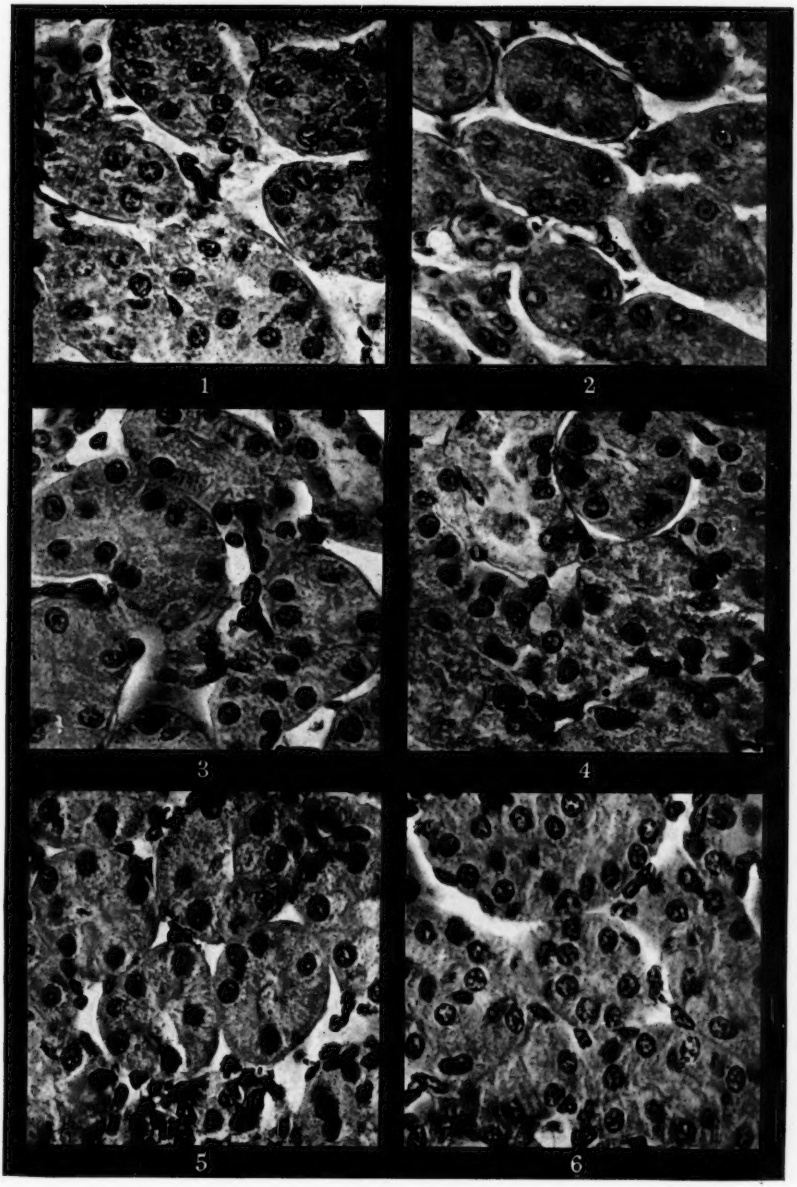
FIG. 2.—Rat kidney 24 hr. after the injection of *S. typhi murium* toxin (4 day M.L.D.), showing proximal convoluted tubules. Feulgen reaction for DNA. (× 520).

FIG. 3.—Rat kidney 24 hr. after the injection of $HgCl_2$ (1 mg./150 g. body-wt.), showing proximal convoluted tubules. Feulgen reaction for DNA. (× 520.)

FIG. 4.—Rat kidney 1½ hr. after the ligation of the pedicle, showing proximal convoluted tubules. Feulgen reaction for DNA. (× 520.)

FIG. 5.—Normal proximal convoluted tubules of guinea-pig kidney. Feulgen reaction for DNA. (× 520.)

FIG. 6.—Guinea-pig kidney 24 hr. after the injection of diphtheria toxin (1 M.L.D./250 g. body-wt.), showing proximal convoluted tubules. Feulgen reaction for DNA. (× 520.)



Fonnesu and Severi.

The RNA content of the renal cells in cloudy swelling produced by bacterial toxins did not differ significantly from that of the controls, nor did the appearance of the sections stained for RNA by Brachet's method suggest a diminution in this substance.

On the other hand, in the kidneys damaged by mercuric chloride poisoning or by the ligation of the renal pedicle (Table II) the chemical determinations showed a significant diminution in both DNA and RNA. Histological examination of these kidneys revealed several areas of necrosis, in which, according to the histochemical tests, both DNA and RNA either disappeared completely or were much reduced. In areas not affected by necrosis the nuclei stained only slightly less intensely than normal, and the staining for RNA was unchanged. Thus the greater decrease of DNA and RNA observed chemically must be attributed to disappearance of nucleic acids from the necrotic areas.

These results suggest to us that the classical picture of cloudy swelling may only be produced with bacterial toxins, since the renal injury caused by mercuric chloride or by the ligation of the renal pedicle represents a more severe pathological condition. Other investigations indicate differences between the states of cloudy swelling produced by bacterial toxins on the one hand and by chemical poisons or by mechanical means on the other. Fonnesu (1952, 1953) found that the uptake of oxygen and the oxidation of fatty acids always decreased in cloudy swelling, and that this decrease was greater in kidneys damaged by mercuric chloride or by renal pedicle ligation than in kidneys injured by the injection of *S. typhi murium* or diphtheria toxins. Further, Fonnesu and Severi (1952) observed in histochemical studies that after the injection of bacterial toxins the alkaline phosphatase in the tubular cells of the kidney diffused throughout the cytoplasm, and was not localized to the brush border and near the basal membrane as in the normal organ. In the kidney of animals poisoned with mercuric chloride or submitted to renal pedicle ligation, except for the necrotic parts which stained diffusely, the alkaline phosphatase showed a normal distribution.

Our present findings, therefore, suggest that the term "cloudy swelling" comprises a rather heterogeneous group of conditions in which cellular swelling is the only common feature.

It is difficult to decide how DNA diminishes, and what consequence its diminution may have on the cellular economy. A high nucleic acid content is known to occur in cells in which protein synthesis is very active. Recently Dounce (1952) proposed a hypothetical mechanism for peptide chain and nucleic acid synthesis. According to this hypothesis nucleic acid would function as a template or distributing agent in governing the order of arrangement of amino acids in peptide chains as well as the order of nucleotides in nucleic acids. With this in view it appears reasonable to assume that the increased protein content observed in cells in cloudy swelling and considered typical of this cellular metamorphosis (Hoppe-Seyler, 1921, 1923, 1928 ; Domagk, 1924 ; Groll, 1927*a*, *b* ; Fricke, Groll and Meyer, 1929; Popják, 1948) is not due to an enhancing of the peptide chain synthesis but is rather a phenomenon of storage, in agreement with Zollinger's (1950) observations.

SUMMARY.

Cloudy swelling of the kidney has been produced in experimental animals by chemical and mechanical methods.

No increase in the water content of such kidneys could be demonstrated.

By histochemical and chemical methods it was found that a diminution of both DNA and RNA, or of DNA only, depended on the mode of production of the cloudy swelling.

The results have been analysed statistically and are discussed.

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STUDIES ON O/R POTENTIAL, pH AND PROTEINASE PRODUCTION IN CULTURES OF *STREPTOCOCCUS PYOGENES*, IN RELATION TO IMMUNIZING ACTIVITY.

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It is well established that a type-specific immunity against infection with *Streptococcus pyogenes* may be induced in mice by inoculation with heat- or acetone-killed vaccines of mouse-virulent strains (Lancefield and Todd, 1928; Stamp and Hendry, 1937; Smolens and Warner, 1952). There is considerable evidence, however, from unpublished experiments, that the degree of protection conferred is very variable, and depends partly on the strain used, and also with some strains on the batch of digest broth in which the vaccines are grown. An explanation of these observations has been sought in the present paper.

In the first experiments, the O/R potential of cultures, which Hewitt (1930, 1950) has shown varies considerably with different batches of medium, was studied in relation to the immunizing activity of the heat-killed organisms. Vaccines were grown at low potentials by incorporating reducing substances in the medium and at high potentials by aeration brought about by shaking in air or oxygen, and also by incorporating 50 per cent serum in the medium (Hewitt, 1930). The immunizing activity of the vaccines was found to vary significantly when grown at different O/R potential levels in some but not all batches of medium, but particularly in those producing a relatively low final pH. It seemed that this might be due to the activity of streptococcal proteinase. This extra-cellular enzyme, present in culture filtrates of many strains of *Str. pyogenes* (Elliott, 1945, 1947, 1950; Todd, 1947), was shown by Elliott to be capable of destroying the serological reactivity of the " M " protein substance in bacterial cells or their extracts. It was produced in active form in the majority of types of medium tested, but in some it was either absent in demonstrable amounts, or present only in the form of an inactive precursor which could be activated autocatalytically or by treatment with trypsin or sodium thioglycollate. It was produced in optimal amounts at pH 5·5–6·5 but not in demonstrable amounts at a neutral or alkaline pH, and was active only under reducing conditions in the presence of sulphydryl groups.

Accordingly the present work was extended (1) to determine the relationship between proteinase production and immunizing activity, using different strains and batches of digest broth; (2) to confirm Elliott's observations regarding the conditions necessary for the optimal production and activation of the enzyme, using the milk clotting test (Elliott, 1947); (3) to study the effect on immunizing activity of incorporating active filtrates in the medium in which the vaccines were grown or of allowing them to act on heat-killed vaccines at varying pH;

and (4) to determine whether immunization with proteinase or its precursor afforded any protection.

MATERIALS AND METHODS.

Strains of streptococci.

These are given in Table VII together with their approximate average lethal doses (A.L.D.) for mice. They had been obtained originally from Drs. F. Griffiths, L. Colebrook, E. W. Todd and A. W. Stableforth, and passaged 20–40 times before being dried in mouse spleens over P_2O_5. They were preserved by this method for about nine years and thereafter in gelatin-ascorbic acid (Stamp, 1947). Strain "Richards" was used for studying the influence of O/R potential and other growth conditions on immunizing activity. Before use each strain was passaged once or twice through mice.

Culture media.

Horse flesh or beef digest broth was used in all experiments except those recorded in Table I, in which the vaccines were grown in a mixture of equal parts of horse digest broth (Batch A) and Lemco broth. This medium gave a heavy grey growth in static culture, and a final pH of *ca.* 6·0. The method of preparation of digest media was essentially that described for Hartley's broth (Mackie and McCartney, 1948). The following other batches of medium were used: Batch B (Table II) prepared from beef flesh digested for 5 hr. at 37° with commercial pancreatin (5 units (Cole) per g. of meat). It gave a moderately heavy grey-black growth in static culture, a final pH of 7·2–7·4 and a relatively low O/R potential. C18 (Tables III and VII) was prepared from horse flesh digested for 18 hr. It gave a heavy whitish growth on static culture, a final pH of 7·2 and a relatively low O/R potential. A further batch (C6) prepared from the same sample of horse flesh but digested for only 6 hr. was used for the preparation of vaccine of strain "C203" (Table VII). It differed from C18 in giving a relatively high O/R potential with strain "Richards."

Batches D2–D24 (Table IV) were prepared from a sample of horse flesh digested for varying periods. Growth was moderately heavy, whitish in colour, the final pH was relatively low and the O/R potential levels variable.

Batch E used for strain S34 vaccines (Table VII and VIII) and the "Richards" vaccines (Table IX) was prepared from horse flesh digested for 5 hr. It gave a moderate white growth on static culture, a final pH of 7·2 and a relatively low O/R potential with strain "Richards."

Batch F used for the production of proteinase precursor and proteinase (Tables V, VI and VIII) was prepared from horse flesh digested for 6 hr. at 37°. It gave a very heavy white growth, a final pH of 6·0 and a relatively high O/R potential.

Batch G used for the production of proteinase precursor (Table IX) was prepared from horse flesh and reinforced with 20 per cent liver digest.

Culture vessels.

In early experiments the medium for static cultures was distributed in 450 ml. quantities in 500 ml. flasks (Table I). Na ascorbate (a 10 per cent solution of ascorbic acid sterilized by boiling and neutralized with 20 per cent NaOH) was added to some to a concentration of 0·1 or 0·25 per cent after autoclaving. The medium for shaken cultures was distributed in 120 ml. quantities in 700 ml. screw-capped bottles.

In all later experiments (Tables II, III, IV, VII and VIII) in which O/R potential readings were taken throughout growth, the medium for static cultures was distributed in 150 ml. quantities in test-tubes 5 × 20 cm. with a capacity of about 220 ml. Na thioglycollate (Judex chemicals) was used as reducing agent in concentrations of 0·25 and 0·1 per cent and was added just before autoclaving. The medium was cooled down rapidly after autoclaving and then inoculated. The test-tubes used were stoppered with a plug of cotton-wool through which were passed four short lengths of glass tubing also plugged with cotton-wool and held in place by a perforated bakelite disc. Two of these were for supporting the electrodes, one for supporting the agar bridge, and the fourth for introducing the inoculum. A circular bakelite stand having a central glass receptacle filled with saturated KCl was used to support up to eight test-tubes.

The medium for shaken cultures or cultures aerated by mixing was distributed in 100 ml.

amounts into cylindrical glass vessels 5 (int.) × 30 cm. with a capacity of about 500 ml. The neck of the vessel was narrowed to take a 2·5 cm. rubber bung, through which passed a single electrode and an agar bridge. When it was necessary to evacuate the bottle and replace the air with oxygen the bung was also fitted with a wide bore needle to which was attached a short piece of rubber tubing fitted with a clip. After the medium had been inoculated the bung was held firmly in position by means of a metal clamp. Before evacuating a bottle, the outer end of the agar bridge was sealed by dipping into melted black pitch at a suitable temperature, in order to avoid sucking the agar into the bottle. The bottle was evacuated by means of a water pump until the residual pressure was about 100 mm. Hg. Oxygen was then run in until pressures had equalized. This procedure was repeated once. The seal on the outer end of the agar bridge was then broken off. The vessels were attached horizontally by means of clips to a board fitted to the shaking machine or horizontally to a slowly revolving drum when less violent mixing was required.

Inoculum.

The inoculum for each culture was usually one part of a 24-hr. digest broth culture to 200 parts of medium.

Method of recording O/R potentials.

Oxidation reduction measurements were made in the conventional manner essentially as described by Knight (1930) and Hewitt (1950), except that a Muirhead pH meter, type D303–B, was used as potentiometer. Certain technical modifications were also necessary owing to the fact that aeration was effected by rotating or shaking the tubes rather than by bubbling air. The electrodes consisted of glass tubes about 3 mm. (int.) × 37 cm. filled with mercury. A 2 cm. piece of platinum wire (s.w.g. 26) was fused in one end so that about 1 cm. projected externally. For static cultures the upper end was left open for the introduction of the copper wire lead. For shaken cultures a short piece of covered copper wire with the ends exposed was sealed with black pitch into the upper end of the electrodes, care being taken as far as possible to avoid air bubbles. Before sterilizing, electrodes for static cultures were passed into glass tubing of slightly wider bore plugged at the bottom with cotton-wool and sterilized in an upright position by dry heat. The electrodes of shaken cultures together with the agar bridge were passed through the rubber bung referred to above and were protected from contamination in a similar way and sterilized by autoclaving. The sterile melted agar-KCl solution was then introduced into the bridge by suction under aseptic conditions. After the medium had been inoculated, the bridges and electrodes were removed from their protective glass tubing and introduced into static cultures by passing through the glass tube supports in the cottonwool plug, and into shaken cultures by fixing the rubber bung in position. Two electrodes were used for each static culture. In earlier experiments the tip of one was placed about 2 cm. from the bottom of the culture and that of the other the same distance from the surface. In later experiments and in all shaken cultures the tips of the electrodes were adjusted to a position midway between the surface and the bottom of the cultures. The static cultures were then placed in the circular stand with the outer ends of the agar bridges dipping into a central reservoir containing saturated KCl solution. The standard half cell, a saturated calomel electrode, was also immersed in this receptacle. Covered wire leads from the mercury of each electrode were carried through the wall of the 37° incubator room to an external switch board and thence to one terminal of the potentiometer. The other lead from the standard half cell was carried similarly to the other terminal, a switch being provided to reverse the connections if required. O/R potential readings were taken on the static cultures *in situ*. The shaken cultures were first removed from the shaking machine and the wire leading from the electrode was then dipped into a small vessel filled with mercury, which was connected with one terminal of the potentiometer *via* the switch-board by a similar wire lead. The agar bridge was dipped into the KCl reservoir. Care was taken to avoid touching bare leads. O/R potential readings were usually taken every 2 hr. for the first 12 hr., and then once or twice the following day before harvesting the growth. Aerated cultures were shaken for 5 min. before initial readings were taken. The average of the readings given by the two electrodes in each static culture was recorded. E.M.F. readings were referred to the normal hydrogen electrode in calculating Eh values.

Preparation and standardization of vaccines.

After 20–24 hr. growth the cultures were transferred to 250 ml. centrifuge bottles, and centrifuged at 3000 r.p.m. in the cold for about 1 hr. or until the supernatant fluid was clear. The supernatants were then syphoned or decanted and the residual fluid drained off. Samples of each supernatant were set aside for measurements of final pH. In most experiments this was done by means of the Muirhead pH meter. The deposits of each culture were then re-suspended in approximately 6 ml. of sterile distilled water. Of this thick suspension 1 ml. was transferred into a weighed centrifuge tube and the remainder into a sterile test-tube. The latter was then plated out for purity and heated at 55° for 1 hr. It was then re-plated to test for sterility and stored at 0° without a preservative under a rubber cap seal. The suspensions were standardized on a dry weight basis. Acetone (5 ml.) was added to 1 ml. of thick suspension in the weighed centrifuge tube and centrifuged. The deposit was re-suspended in acetone, re-centrifuged and dried *in vacuo* over P_2O_5. The weight of dry cells per ml. of vaccine was then calculated and the approximate yield per 100 ml. of culture was recorded. Before use the vaccines were suitably diluted to the required concentration with saline.

Preparation of crude proteinase and precursor.

This was done essentially as described by Elliott (1950). Proteinase was isolated from potent culture filtrates of strains "Fipett" and "Usher" after activation with Na thioglycollate by precipitating with 0·8 saturated $(NH_4)_2SO_4$ at 0°, dissolving in a small quantity of distilled water, centrifuging and dialysing the supernatant fluid against tap water followed by distilled water. This procedure was repeated once. The crude proteinase was stored in the cold as a concentrated solution. The amount present was calculated by drying a known volume over P_2O_5. The solution obtained from the "Fipett" filtrate contained 1·2 mg./ml., and the milk clotting titre was 1/1280 or approximately 1000 M.C.U./mg. That prepared from the "Usher" filtrate contained about 300 M.C.U./mg. Before use the solutions were diluted with saline to give a calculated concentration of 0·02 mg./ml.

Precursor was prepared from a culture filtrate of strain "Usher." No attempt was made to purify it by repeated crystallization or to isolate it in a crystalline form. It was stored as a concentrated solution in 0·5 saturated $(NH_4)_2SO_4$ at pH 5·0 at 0°. Titration of a sample of the concentrated solution after activation gave a milk-clotting titre of 1/1600. The unactivated solution was diluted 1/10 in saline immediately before injection into mice.

Estimation of streptococcal proteinase in filtrates.

This was done as described by Elliott and Dole (1947), using a milk-thioglycollate mixture prepared from 50 per cent fresh skimmed milk (Tables V and VI) and 10 per cent suspension of dried skimmed milk powder subsequently. The highest dilution of a filtrate giving rise to clotting after overnight incubation at 37° is referred to as the proteinase titre and as containing 1 M.C.U.

Strains of mice.

Albino white mice weighing usually 20–24 g. were used in most experiments (Tables I, II, III, VII and IX), and C57 black in Tables IV and VIII. The latter strain, particularly the males, was rather more susceptible to infection with *Str. pyogenes*. Batches of 30 mice were used, usually containing equal numbers of males and females, though in some experiments with C57 mice one sex only was used.

Testing of immunizing activity of vaccines.

Immunization was by the intraperitoneal route, two doses of 0·1 mg. usually being given 1 week apart, followed by a challenge dose of living organisms 1 week later. This consisted of approximately 100 A.L.D. contained in 0·5 ml. of a 1/100 or 1/1000 dilution of a washed serum broth culture. Mice were kept under observation for 14 days after infection, the deaths being recorded daily.

RESULTS.

The Effect of Varying O/R Potential on Immunizing Activity.

In the first three experiments the vaccines of strain " Richards " were grown at varying oxygen tensions in a mixture of Hartley and Lemco broth. The final pH of the static cultures was 6·0–6·4, and that of the aerated cultures was 6·8. A marked feature was the black colour of the suspensions grown under static conditions. The aerated cultures yielded a white growth. Some of the vaccines were stored in the presence of a reducing agent. In the first experiment the mice received 3 weekly doses of 0·25 mg. instead of the customary 2 doses of 0·1 mg.

TABLE I.—*Immunizing Activity of Vaccines of* Str. pyogenes (" *Richards* ") *Grown in Hartley-Lemco Broth at Varying Oxygen Tensions and the Effect of Preserving in the Presence of a Reducing Agent.*

Conditions of growth.	Protective effect of vaccine in mice.			
	Number of mice per batch.	Number of survivors.	Per cent survival.	Standard error.
Experiment 1 :				
*Anaerobic jar + Na ascorbate 0·1 per cent	30	6	20·0	7·3
*No jar—Na ascorbate 0·1 per cent	30	4	13·3	6·2
*Normal static	30	10	33·3	8·6
Normal static	30	14	46·7	9·1
Shaken air	30	26	86·7	6·2
No vaccine given	30	1	3·3	3·1
Experiment 2 :				
Anaerobic jar + Na ascorbate 0·1 per cent	30	0	0·0	..
Normal static	30	11	36·7	8·8
Shaken air	30	20	66·7	8·6
Shaken O_2	30	22	73·3	8·1
No vaccine given	30	1	3·3	3·1
Experiment 3 :				
Na ascorbate 0·1 per cent	30	0	0·0	..
Na ascorbate 0·05 per cent.	30	1	3·3	3·1
Normal static	30	2	6·7	4·6
Shaken air	30	14	46·7	9·1
Shaken O_2	30	15	50·0	9·1
No vaccine given	30	0	0·0	..

* Preserved in the presence of Na ascorbate 0·1 per cent.

It will be seen that the immunizing activity of the vaccines varies significantly. In all three experiments the most potent vaccines are those grown under raised oxygen tension, and the least active those grown under anaerobic conditions. The use of an anaerobic jar, in addition to the incorporation of Na ascorbate in the medium for maintaining anaerobic conditions and also the use of Na ascorbate as a preservative, did not appear to produce any significant modification in activity. These procedures were therefore discontinued in subsequent experiments. It was also found that the least active vaccines were those from the cultures developing the lowest final pH. The next set of vaccines were grown in

a batch of digest broth (Batch B) in which little or no change of pH occurred after 24 hrs.' growth, in order to investigate the possibility that variations in immunizing activity were due to differences in pH rather than in O/R potentials. Measurements of O/R potential levels were made during growth (Figure). The methods used to vary the O/R potential were similar to those used previously, except that Na thioglycollate was substituted for the Na ascorbate as reducing agent, and 50 per cent serum cultures shaken in O_2 were included in the series in order to obtain as high a level as possible. The cultures were put up in duplicate, one of each pair being fitted with electrodes and agar bridge. The O/R potentials

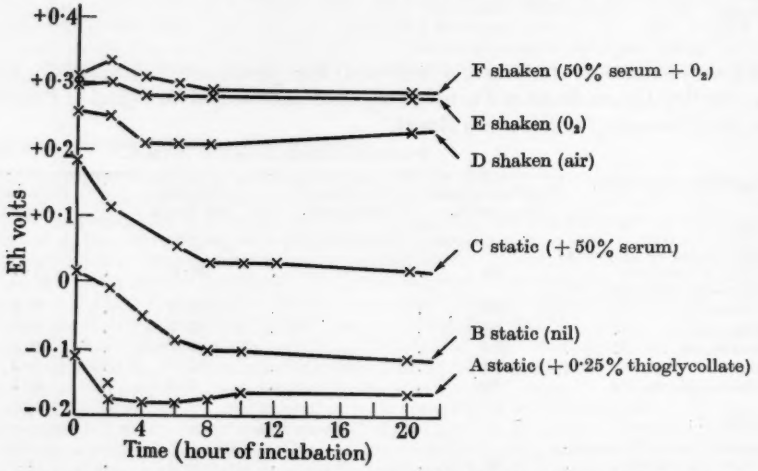

FIGURE.—The O/R potentials recorded in digest broth (Batch B) cultures of *Str. pyogenes* grown under varying conditions of oxygen tension.

recorded resemble fairly closely those given by Hewitt (1950) for *Str. pyogenes* in digest broth. The initial O/R potential level in normal static cultures depends partly on the interval between autoclaving and inoculation. In this experiment the static cultures were inoculated immediately after cooling rapidly and the levels are therefore relatively low. The 24-hour readings of the aerated cultures are not as high as those recorded by Hewitt, which reached a figure of +0·4 vt. This was probably due to the batch of medium used, since in a subsequent experiment with a different batch that figure was recorded in the culture shaken with oxygen. The protective effect of each pair of vaccines was tested on batches of 60 mice, 30 receiving the vaccine from the cultures in which O/R potential readings had been taken and the others being immunized with the duplicate. The immunizing procedure was three doses of 0·1 mg. at weekly intervals. The results are given in Table II.

It will be seen that the average level of immunity is much higher than in the previous tests and the differences in immunizing activity, though significant and related to the mean O/R potential levels, are not as marked; when the test was repeated using the usual two weekly doses of vaccine, greater differences in

TABLE II.—*The Effect of Immunization with Vaccines of* Str. pyogenes (*"Richards"*) *Grown in Beef Digest Broth (pH 7·8) with Low O_2-Carrying Capacity at O/R Potentials Recorded in Figure.*

Batch of vaccine.	Conditions of growth.	Final pH.	Yield (mg./100 ml.).	1st experiment. Immunizing dose = 0·1 mg. × 3. Number of mice.	Number of survivors.	Per cent survival.	Standard error.	2nd experiment. Immunizing dose = 0·1 mg. × 2. Number of mice.	Number of survivors.	Per cent survival.	Standard error.
A	+0·25 per cent thioglycollate	7·5	14	60	39	65·0	6·2	40	4	10·0	4·7
B	*Nil* static standard	7·7	19	60	48	80·0	5·2	39	4	10·3	4·8
C	*Nil* static + 50 per cent serum	8·4	33	60	51	85·0	4·6	38	11	29·0	7·4
D	Shaken—air	7·6	31	60	54	90·0	3·9	40	21	52·5	7·9
E	Shaken O_2	7·8	33	60	52	86·7	4·4	40	5	12·5	5·2
F	O_2 (50 per cent serum)	8·2	56	30	17	56·7	9·0	—	—	—	—
Control	No vaccine given	—	—	30	0	0·0	—	40	0	0·0	—

TABLE III.—*Immunizing Activity of Vaccines of Three Different Strains of* Str. pyogenes *Grown at High and Low O/R Potentials in a Batch of Non-Acid producing Digest Broth (C18).*

Strain.	Conditions of growth.	O/R potential range—vt. 2 hr.	24 hr.	Final pH.	Yield (mg./100 ml.).	Number of mice.	Number of survivors.	Per cent survival.	Standard error.
Richards	0·25 per cent thioglycollate	−0·12	−0·14	7·0	28	30	24	80·0	7·3
	Shaken air	+0·22	+0·22	7·1	44	30	23	76·7	7·7
	Control—no vaccine	..	..	..	..	30	0	0	..
William	0·25 per cent thioglycollate	−0·12	−0·13	7·1	24	30	7	23·3	7·7
	Shaken air	+0·2	+0·14	6·9	58	30	5	16·7	6·8
	Control—no vaccine	..	..	..	..	30	2	6·7	4·6
Gillard	0·25 per cent thioglycollate	−0·11	−0·14	7·0	23	30	6	20·0	8·6
	Shaken air	+0·22	+0·14	7·0	64	30	3	10·0	5·5
	Control—no vaccine	..	..	..	..	30	2	6·7	4·6

immunizing activity between those grown at high and low O/R potentials were demonstrated, suggesting that variation in O/R potential rather than pH was mainly responsible for the results recorded in Table I.

In a further experiment, however, in which another batch of non-acid-producing medium (C18) was used, vaccines of strain " Richards " showed no significant difference in immunizing activity when grown at widely different O/R potential levels (Table III). Vaccines prepared from two other strains under the same conditions showed little or no immunizing activity even when grown at high O/R potentials. These strains were investigated further in Table VII.

It seemed that the difference in the results obtained by varying the batch of medium might in part be due to difference in the degree of digestion of the meat with trypsin. This possibility was tested in the next experiment. A batch of horse flesh was minced and divided into six equal portions. These were digested under the same conditions for varying periods. Vaccines were prepared from each of these batches and tested in the usual way in two experiments. In the first they were grown in normal static culture, and in the second Na thioglycollate (0·1 per cent) was added to the medium.

O/R potential levels were recorded throughout growth on duplicate sets of cultures. No consistent differences were produced by altering the period of digestion. In the first experiment the initial O/R potentials of the normal static cultures D2 to D18 were somewhat higher than those recorded in Table I (i.e., +0·18 to +0·16 vt). The levels fell more rapidly and reached a lower minimum figure of −0·12 to −0·2 vt in 4–6 hr., rising to about −0·1 vt at the end of the growth period. In the 24-hr. digest broth culture there was an initial reading of +0·24 vt, a delayed fall, a higher minimum figure of −0·1 vt and a marked rise after 22 hr. to +0·05 vt, suggesting greater oxygen carrying and poising capacity. In all the cultures containing Na thioglycollate very low O/R potentials varying from −0·16 to −0·28 vt, were recorded. The results of immunization are given in Table IV.

It will be seen that no significant difference in immunizing activity is brought about by varying the period of digestion. Furthermore, with these batches of

TABLE IV.—*Results of Immunization with Vaccines of Strain " Richards " Grown in Horse Flesh Digest Broth Digested with Trypsin for Varying Periods, and the Effect of Adding Na thioglycollate to the Medium.*

Period of digestion (hr.).	Thioglycollate 0·25 per cent.	Final pH.	Yield (mg./100 ml.).	Number of mice.	Number of survivors.	Per cent survival.	Standard error.
2	—	*6·4 (6·2)	21	30	22	73·3	8·1
4	—	6·4 (6·25)	23	30	22	73·3	8·1
6	—	*6·4 (6·3)	34	30	25	83·3	6·8
12	—	*6·4 (5·9)	34	30	20	66·7	8·6
18	—	*6·5 (6·7)	26	30	20	66·7	8·6
24	—	*6·5 (6·8)	23	30	19	63·3	8·8
Control	..	..	..	30	0	0	..
2	+	6·4	15	30	6	20·0	7·3
4	+	6·3	20	30	15	50·0	9·1
12	+	6·6	27	30	12	40·0	9·0
18	+	6·5	28	30	17	56·7	9·0
24	+	6·5	26	30	13	43·3	9·0
Control	..	..	..	30	1	3·3	3·1

* Adjusted by addition of acid or alkali after 12 hr. growth to bring all to a final pH of 6·4–6·5. Figures in parentheses refer to final pH of an unadjusted series.

medium a relatively high degree of immunizing activity resulted even when a very low O/R potential was maintained throughout growth, and the final pH was also relatively low. It was clear therefore that these factors were not the only ones concerned in influencing immunizing activity.

It seemed that these results might well be due to the activity of proteinase produced in the vaccine culture. With many batches of medium the most marked reduction in immunizing activity occurred when the vaccines were grown at a low O/R potential and pH, conditions which favour the production and activation of proteinase, while the immunizing potency was unimpaired at high O/R potentials, when proteinase is inactive. The fact that this phenomenon can only be demonstrated in certain batches of medium is also in accord with Elliott's observations that proteinase is only produced in some types of medium (Elliott, 1947).

In the next experiments therefore the proteinase-producing capacity of a number of strains was studied using the milk-clotting test described by Elliott and Dole (1947), and this was compared with the ability of these strains to induce an effective immunity.

The Proteinase-Producing Ability of Different Strains of Str. pyogenes.

Twenty mouse-virulent Group A strains and one of group C were tested for their ability to produce acid and clot in litmus milk. Some gave a positive result in 2–3 days, others within 10 days, and a few were negative after 14 days. Nine strains selected from each of these groups were investigated further for their ability to produce a milk-clotting enzyme in culture filtrates.

One of the most active strains (Fipett) was grown in different batches of horse digest broth giving a final pH of *ca.* 6·0. After 24 hr. the cultures were centrifuged and the supernatant fluids filtered through a Seitz pad. The filtrates after standing overnight at 18° were tested for milk-clotting activity. The most active filtrate produced a clot after 18 hr. in a dilution of 1/16. The medium from which this filtrate was derived was therefore used to prepare filtrates of all nine strains under investigation. These were kept at 20° for three days before being tested. The activity of the filtrates at varying pH was tested by adjusting samples to the required pH with N HCl or N NaOH, making serial dilutions in saline, and adding a 50 per cent milk-phosphate-thioglycollate mixture adjusted to the same pH. The results are given in Table V.

It will be seen that the culture filtrates vary greatly in milk-clotting activity when tested under these conditions, those of the three strains "Richards," "C203" and "S34" failing to show any activity. The activity of the other strains varied somewhat with the pH, though in no case was the titre determined owing to the fact that activity was much greater than had been anticipated from the results of the preliminary test with "Fipett" filtrates. The reason for this was the much greater degree of conversion of precursor to proteinase that occurred with these filtrates. This phenomenon was investigated further in the experiment recorded in Table VI.

It will be further noted that milk-clotting activity is most pronounced at pH 8·0, falling off markedly at pH 7·0 and then apparently increasing again at pH 6·0. The latter increase may be more apparent than real, and due to the increased sensitivity of the test as the pH approaches 5·5, where spontaneous

TABLE V.—*The Milk-Clotting Activity of Culture Filtrates of Different Strains of* Str. pyogenes *at Varying pH.*

pH of milk/thioglycollate mixture.		Fipett.					E14.					Usher.				
		6·0	6·5	7·0	7·5	8·0	6·0	6·5	7·0	7·5	8·0	6·0	6·5	7·0	7·5	8·0
Final dilution of filtrate	1/2	15	15	15	10	15	10	20	15	10	10	20	25	25	35	—
	1/4	30	40	50	50	60	30	40	40	30	30	35	35	75	150	—
	1/8	50	75	240	50	60	50	50	150	75	75	50	180	180	180	—
	1/16	90	300	—	300	75	90	300	—	150	75	75	300	300	180	—
	1/32	240	—	—	300	90	205	—	—	205	90	105	300	—	105	120
	1/64	—	—	—	—	90	300	—	—	205	90	180	—	—	105	105

pH of milk/thioglycollate mixture.		William.					Gillard.					Schoenhoyher (1039).				
		6·0	6·5	7·0	7·5	8·0	6·0	6·5	7·0	7·5	8·0	6·0	6·5	7·0	7·5	8·0
Final dilution of filtrate	1/2	17	25	35	75	—	15	20	25	75	—	300	—	300	—	90
	1/4	50	60	135	135	—	50	55	240	300	—	300	—	—	—	135
	1/8	60	135	300	180	—	105	135	240	210	—	—	—	—	—	180
	1/16	105	300	—	120	105	120	240	—	135	210	—	—	—	—	300
	1/32	—	—	—	240	120	180	—	—	120	120	—	—	—	—	—
	1/64	—	—	—	135	120	300	—	—	120	120	—	—	—	—	—

pH of milk/thioglycollate mixture.		Richards.					C203.					S34.				
		6·0	6·5	7·0	7·5	8·0	6·0	6·5	7·0	7·5	8·0	6·0	6·5	7·0	7·5	8·0
Final dilution of filtrate	1/2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1/4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1/8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Figures = time in min. for visible clotting to take place. Observation period, 5 hr. — = no clot.

clotting due to precipitation of the caseinogen occurs. Alternatively it may be that two enzyme systems with different optimal pH are concerned in this reaction.

It will be seen also that inhibition of milk-clotting activity occurs at low dilutions and an alkaline pH with some but not all of the active filtrates. The reason for this is not clear, but it may be noted that a similar effect occurs with other enzyme systems such as the R.D.E. of the cholera vibrio acting on non-specific haemagglutination inhibitor (Isaacs and Bozzo, 1951). These workers also found that the prozone occurs inconstantly.

Partial clearing of the milk often occurred in the tubes showing a prozone. This may be due to activity of another enzyme present in the filtrate.

The Conversion of Precursor to Proteinase.

The differences in milk-clotting titre which had been noted in the culture filtrates of strain " Fipett " when kept for different periods before being tested, presumably due to conversion of precursor into active proteinase, was investigated further. Culture filtrates of two strains, " Usher " and " Gillard," were prepared in the batch of medium used previously under conditions producing a high O/R potential (+0·1–+0·01 vt), i.e., in shallow layers of medium which had been autoclaved 48 hr. before use. After 18 hr. growth the culture was centrifuged, filtered, and samples tested the same day. The results of this preliminary titration were identical with those subsequently obtained with samples stored at 0° for 3 days. Samples were stored under varying conditions for 3 days, then adjusted to pH 8·0 if necessary, diluted in 0·067 M phosphate buffer and mixed with milk-thioglycollate mixture (pH 8·0) as before (Table VI).

It will be seen that most of the proteinase is in the form of inactive precursor when stored at 0° and 20°. It is rapidly converted into active proteinase by Na thioglycollate at an acid pH, but less completely in an alkaline. As Elliot found, Na thioglycollate not only activates precursor, but also sensitizes the milk-clotting reaction. It will be noted that no prozone developed in this test.

The Effect of Varying the Period of Digestion of Broth with Trypsin on Proteinase-Producing Capacity.

Strain " Fipett " was grown for 24 hr. in the batches of digest broth referred to in Table IV. After 11 hrs.' growth the pH was adjusted with 0·1 N HCl or NaOH to produce a final pH of about 6·0, the approximate amounts required having been determined by preliminary tests. The final pH of the cultures varied from 5·8 to 6·1. The culture filtrates were activated overnight with 0·1 M thioglycollate and tested for milk-clotting activity. All gave relatively low titres of 1/10–1/20. These results may be correlated with the relatively high and uniform level of immunizing activity of the vaccines of strain " Richards " tested in Table IV. It also indicates that with the particular sample of horse flesh used, the period of digestion was not important in determining proteinase-producing capacity.

The Effect of Storage of Horse Flesh on the Proteinase-producing Capacity of Digest Broth.

One sample of horse meat fresh from the abattoir was divided into two portions. One was digested immediately, and the other after storage for 3 days

TABLE VI.—*Influence of pH and of Na thioglycollate on the Conversion of Precursor to Active Proteinase.*

Conditions of storage of filtrates for three days.	Final dilution of filtrate (pH 8·0). 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.
"Gillard" at 0°; pH 6·2; no Na thioglycollate	225	280	+	−	−	−	−	−	−
" 20°; " ; " "	215	245	+	−	−	−	−	−	−
" " ; 0·1 M thioglycollate	15	15	20	25	30	40	65	120	+
" " 7·4; no Na thioglycollate	240	270	+	−	−	−	−	−	−
" " ; 0·1 M thioglycollate	30	35	58	80	100	180	+	−	−
"Usher" at 0°; pH 6·2; no Na thioglycollate	250	265	+	−	−	−	−	−	−
" 20°; " ; " "	210	240	+	−	−	−	−	−	−
" " ; 0·1 M thioglycollate	15	20	20	30	30	35	60	110	255
*" " ; " "	+	+	±	−	−	−	−	−	−
" " 7·7; no Na thioglycollate	+	+	±	−	−	−	−	−	−
" " ; 0·1 M thioglycollate	210	225	265	+	−	−	−	−	−

* Titrated with milk containing no thioglycollate.
Figures = time in min. for visible clotting to take place. + = clot present after 20 hr. but not after 5 hr. incubation.

TABLE VII.—*Immunizing Activity of Vaccines of Different Strains of* Str. pyogenes *Grown in Non-Acid Producing Batches of Digest Broth when Challenged with the Homologous Strain.*

Strain.	Group and type.	Proteinase production in acid-producing medium (Table V).	Vaccine culture. Final pH.	Eh range (vt). 4 hr.	22 hr.	Yield (mg./100 ml.).	Immunizing dose (A.L.D. approx.).	Number of mice.	Number of survivors.	Per cent survival.	Standard error.
Fipett	A 3	+++	7·0	−0·1	−0·65	30	100	30	14	46·7	9·1
							controls	30	0	0·0	—
E14	A 10	+++	7·2	−0·1	−0·05	27	1	30	14	46·7	9·1
							control	30	12	40·0	9·0
Usher	A 13	+++	7·1	−0·09	−0·05	29	100	30	0	0·0	—
							controls	30	1	3·3	3·1
William	A 14	+++	6·9	−0·09	−0·16	24	10–100	30	6	20·0	7·3
							controls	30	2	6·7	4·6
Gillard	A 17	+++	6·9	−0·09	−0·11	24	10–100	30	7	23·3	7·7
							controls	30	2	6·7	4·6
Schoenhoyher (1039)	A 9	++	7·2	−0·08	−0·05	19	10–100	30	2	6·7	4·6
							controls	30	8	26·7	8·1
Richards	A 3	−	7·1	−0·09	−0·09	37	100	30	17	56·7	9·0
							controls	30	0	0·0	—
C203	A 1	−	7·2			—	10–100	30	29	96·7	3·1
							controls	30	8	26·7	8·1
S34	C animal	−	7·4			18	100	30	16	53·3	9·1
							controls	30	0	0·0	—

All vaccines grown in Batch C (18) except C203 (C6), and S34 (Batch E + 0·25 per cent Na thioglycollate).

at 4°. A culture filtrate of strain " Fipett " prepared from the former batch had a final pH of about 6·0, and after activation showed moderate milk-clotting activity. A similar culture filtrate prepared from the latter batch in which the yield of growth was less heavy had a final pH of about 7·0 and showed almost no milk-clotting activity. Yield of proteinase in this batch was not improved appreciably by the addition of glucose to increase the growth and bring the final pH to the optimum for proteinase production. This preliminary test suggests that proteinase production may be influenced by labile substances present in fresh horse meat, but rapidly destroyed on storage perhaps as the result of autodigestion. These results, however, require confirmation.

The Immunizing Activity of Vaccines of Different Strains of Str. pyogenes.

The results of immunization with vaccines of three strains grown at high and low O/R potentials are recorded in Table III. It may be noted that the vaccine of the weakly proteinase-producing strain " Richards " when grown at a low O/R potential in broth containing thioglycollate was very active, suggesting that no proteinase was produced by this strain in the medium used. Similar results were obtained with other " Richards " vaccines grown in acid-producing batches, for example D4 and D18 (Table IV). With these media proteinase was demonstrated, though in relatively small amounts in culture filtrates of the strongly proteinase-producing " Fipett " strain. It would appear therefore that with the weakly proteinase-producing strain " Richards," destruction of the immunizing antigen occurs to an appreciable extent during growth even at very low O/R potentials, only in batches of medium strongly favouring the production of proteinase. The vaccines of the strongly proteinase-producing strains " William " and " Gillard " showed no immunizing activity even when grown at a high O/R potential. With these strains it would seem that a sufficient amount of active proteinase is formed to destroy the M substance completely, even when the organisms are grown under conditions relatively unfavourable to proteinase production. It will be seen from Table VI that the " Usher " and " Gillard " culture filtrates grown at a high O/R potential contained a small amount of active proteinase before activation with Na thioglycollate. Furthermore, it has been shown in Table VI and other unrecorded experiments that an active filtrate clots milk to a slight extent even in the absence of Na thioglycollate, i.e., at an O/R potential of about +0·19 vt. It would appear therefore that the change from precursor to proteinase and the ability of the enzyme to clot milk or destroy the M substance is not completely inhibited at high O/R potentials. There is the further possibility that the results in Table III might be due to the fact that with strongly proteinase-producing strains, proteinase or its precursor might exert a marked offensive effect analogous to that produced by the erythrogenic toxin with highly toxigenic strains (Downie, 1930), and that antibodies to one or other of these substances might be necessary for protection. The effect of immunizing with proteinase and its precursor was therefore investigated and the results are recorded in a later section.

In the next experiments vaccines of a number of strains of varying proteinase-producing ability grown under normal static conditions were tested for immunizing activity. They were grown in non-acid producing medium, seven in Batch C18, in view of the marked differences between the activity of vaccines of weakly

animal . . 18 . . 100 controls . . 30 30 . . 16 0 . . 53·3 0·0 . . 9·1 —

All vaccines grown in Batch C (18) except C203 (C6), and S34 (Batch E + 0·25 per cent Na thioglycollate).

and strongly proteinase-producing strains which had been demonstrated with this medium in Table III.

The culture supernatants after centrifuging were filtered, and after activation with Na thioglycollate were tested for milk-clotting activity. All gave a negative result.

The vaccines showed marked differences in immunizing activity, some failing to induce any protection. This could not be correlated with the degree of virulence, which with nearly all strains was of the same order, but was closely related to the ability of the strain to produce the milk-clotting enzyme when grown under suitable conditions (Table V). It would seem therefore that destruction of the M antigen may occur in cultures of strongly proteinase-producing strains even though no milk-clotting enzyme can be detected in the culture filtrate, perhaps as the result of the activity of proteinase bound to the cells.

Immunological tests may therefore be a more sensitive indicator of the production of active proteinase than the milk-clotting test. One strain, "Fipett," however, although a strong proteinase-producer under favourable conditions, nevertheless in the above experiments induced a marked degree of immunity. This result was confirmed with vaccines grown in other batches of non-acid-producing medium. It is possible that for some reason conversion of precursor to proteinase tends to be delayed with this strain, since a batch of vaccine prepared under conditions more favourable for proteinase production and in which proteinase was demonstrated in the culture filtrate showed no immunizing activity. It may be, however, that in spite of the evidence to the contrary (Elliott and Dole, 1947), the immunizing antigen-destroying enzyme and the milk-clotting enzymes are not identical. It will also be noted in Table VII that the rabbit-virulent strain "Usher," which had been found to induce an effective immunity in rabbits (Stamp, 1936), completely failed to immunize mice, thus confirming results obtained sixteen years previously.

The Immunizing Activity of Vaccines Treated with Active Culture Filtrates.

Two experiments were carried out to study the immunizing antigen-destroying activity of culture filtrates at varying pH.

In the first, the proteinase-containing filtrate was allowed to act on the bacterial cells during growth. A culture filtrate of strain "Usher" having a milk-clotting titre of 1/512 was added in a final concentration of 20 per cent to batches of digest broth adjusted to pH 8·0, 7·3, 6·3, and containing 0·25 per cent Na thioglycollate. Cultures containing no filtrate and also one with heated filtrate were also set up. They were inoculated with the weakly or non-proteinase-producing strain "S34," and incubated for 24 hr. The vaccines were prepared and tested as usual and the results are given in Table VIII.

It will be seen that the unheated filtrates have produced a significant reduction in immunizing activity at both pH 6·0 and 8·0. The filtrate heated at 70° for 30 min. was inactive. In the second experiment the filtrate was allowed to act on heat-killed cells of strain "Richards." The bacteria centrifuged from a 24-hr. growth in a non-proteinase-producing batch of digest broth were re-suspended in 0·06 M phosphate buffer containing 0·1 M thioglycollate at pH 6·0, 7·0 and 8·0 to give a calculated concentration of about 12 mg. dried cells/20 ml. "Fipett" filtrate, heated and unheated, was added to 20 ml. quantities, to give a final

TABLE VIII.—*The Effect of Incorporating "Usher" Filtrate (1/5) in Culture Medium + Na thioglycollate (0·25 per cent) Adjusted to Varying pH before Inoculation, on Immunizing Activity of Strain S34 Vaccines.*

	pH range of culture.	Eh range of culture (vt). 4 hr.	24 hr.	Yield (mg./100 ml.).	Number of mice.	Number of survivors.	Per cent survival.	Standard error.
+ filtrate	6·3–5·7	−0·07	+0·07	26	30	5	16·7	6·8
+ filtrate heated 70°	6·3–5·7	—		26	30	13	43·3	9·0
No filtrate	6·3–5·6	—		26	30	10	28·3	8·6
+ filtrate	7·3–6·6	−0·14	−0·025	32	30	9	30·0	8·4
,,	8·0–7·4	−0·14	−0·07	20	30	7	23·3	7·7
No filtrate	8·0–7·4	—		18	30	16	53·3	9·1
Controls	—	—		—	30	0	0	—

concentration of 1/5. The milk-clotting titre at pH 6·0, 7·0 and 8·0 was 1/600, 1/200 and 1/800 respectively. Each suspension was mixed on a rotating drum in a tube closed with a rubber bung for 48 hr. at 37°. The suspensions were then diluted with saline to an estimated concentration of 0·2 mg./ml. and used to immunize batches of mice in the usual way. No attempt was made to remove heated or unheated filtrate from the suspensions. The results are given in Table IX.

TABLE IX.—*The Effect of Treating Heat-killed Vaccines of Strain "Richards" with "Fipett" Culture Filtrate (1/5) at Varying pH.*

Filtrate.	pH of suspension.	Number of mice.	Number of survivors.	Per cent survival.	Standard error.
Unheated	6·0	30	1	3·3	3·1
Heated 70°	6·0	30	10	33·3	8·6
Unheated	7·0	30	2	6·7	4·6
Heated 70°	7·0	30	13	43·3	9·0
Unheated	8·0	30	6	20·0	7·3
Heated 70°	8·0	30	6	20·0	7·3
Controls	—	30	2	6·7	4·6

The filtrate produced a significant destruction of the immunizing antigen at pH 6·0 and 7·0 but, unlike the previous experiment, not at pH 8·0. At this pH both treated and untreated cells are relatively inactive.

The Effect of Immunizing with Crude Proteinase or its Precursor.

An experiment was carried out to determine whether immunization with crude proteinase, administered either alone or in combination with the inactive vaccines used in Table VII, was capable of conferring protection. Crude proteinase prepared as described above was given subcutaneously in two doses of 0·01 mg. (or approximately 20 M.C.U. of "Fipett" proteinase, and 7 M.C.U. of "Usher" proteinase) per mouse. Vaccines were given by the intraperitoneal route. The strains used for challenge were strong proteinase-producers, namely "Usher," "Fipett," "1039" and "Gillard." No significant protection was conferred by immunization with proteinase alone or in combination with vaccines against infection with homologous or heterologous strains. In another experiment crude precursor also failed to induce any immunity in the dosage employed.

DISCUSSION.

These experiments show that the immunizing activity of vaccines of *Str. pyogenes* depends on a number of factors related to the production of streptococcal proteinase, which Elliott (1945) showed was able to destroy the serological reactivity of the M antigen.

In the first place the strain used is of importance. Not all mouse-virulent strains are capable of inducing an effective immunity in mice against infection with the homologous strain. Vaccines prepared from strongly proteinase-producing strains, with one exception, namely "Fipett," show little or no immunizing activity even when grown under conditions unfavourable for the production and activation of proteinase in culture filtrates. It may be that under such conditions proteinase, though not demonstrable in culture filtrates, is present in the bacterial cell in sufficient amount to bring about destruction of the immunizing antigen. Further examination of these vaccines for the presence of M antigen by serological methods is required to test this hypothesis. Alternatively, it is possible that anti-M antibodies are of less importance in protecting against these strains, and that antibodies against another unidentified antigen are necessary. The possibility that this antigen might be proteinase itself or its precursor has been investigated, but immunization with crude preparations of these substances has given negative results. It is interesting to note that heat-killed vaccines of one strongly proteinase-producing strain, namely "Usher," which consistently failed to protect mice, induced an effective protection in rabbits (Stamp, 1936), indicating that different antigens may be concerned in inducing protective antibodies in these two animal species against infection with this organism.

Vaccines prepared from weakly proteinase-producing strains such as "Richards," "C203" and "S34" have given variable results, depending on the conditions under which the vaccines are grown. The batch of medium used, the O/R potential and pH developed during growth have all been found to influence immunizing activity. A great reduction in immunizing activity results when vaccines are grown in certain batches of medium giving a final pH of *ca.* 6·0, particularly when reducing substances are incorporated. Though little or no proteinase may be demonstrated in filtrates of these cultures, parallel tests of culture filtrates of strongly proteinase-producing strains have confirmed Elliott's observation that these conditions are also most favourable for production and activation of proteinase. It would seem most likely therefore that the variation in immunizing activity noted with weakly proteinase-producing strains is due to destruction of the immunizing antigen by proteinase produced during the growth period. The factors in the medium which influence the formation of precursor and its conversion to proteinase have not yet been fully determined. Elliott and Dole (1947) noted great variation in the yields of proteinase produced in different media, but this appeared to be due to the fact that in some the enzyme remained in the precursor form. In others, made from batches of peptone containing traces of trypsin, it was rapidly converted into active proteinase.

Olarte (1948) was unable to demonstrate any proteinase in his culture filtrates, but this was probably due to the fact that acid produced by the fermentation of glucose was neutralized at intervals and the acid pH necessary for liberation of precursor from the cells was not present (Elliott, 1950).

In the present experiments horse flesh or beef digest media were used exclu-

sively. Marked variations in the final pH were recorded. The reasons for this are not clear. The presence of fermentable carbohydrate such as glycogen in varying amounts may have been partly responsible. Haemolytic streptococci may also produce acid in carbohydrate-free media, presumably splitting it off from the carbohydrate radicles present in the proteins (McLeod, 1929). An experiment recorded above suggests that one substrate may be very labile and be destroyed as the result of autodigestion. The relatively high pH produced in shaken cultures may have been due to removal of CO_2 from the cultures. Apart from an acid pH a high yield of precursor and subsequently of proteinase was constantly associated with an abundant growth of organisms. Little or no proteinase could be demonstrated in filtrates of cultures yielding only a moderate or poor growth even though O/R potential and pH conditions were favourable. The fact may possibly have some bearing on the observations of Belton and Strange (personal communication) on the immunizing activity of vaccines of *Bacillus anthracis* prepared from a " non-proteinase-producing " variant. They noted that vaccines prepared from cultures yielding a heavy growth were relatively inactive. It may be that this strain resembles the weakly proteinase-producing strains of *Str. pyogenes* used in the present work in producing sufficient proteinase to bring about a marked destruction of the immunizing protein, only under particularly favourable conditions associated with a heavy yield of organisms.

It would seem that other factors also influence the formation of precursor, since there may be considerable variation in the amount produced in different batches of medium, when they are adjusted to give a similar yield of cells and final pH by the incorporation of the requisite amount of glucose. The preliminary tests referred to above indicated that one such factor may be rapidly destroyed as a result of autolytic processes occurring in meat on storage even at low temperatures.

The conversion of precursor into active proteinase may be brought about by trypsin or autocatalytically in the presence of Na thioglycollate (Elliott, 1950). The importance of O/R potential in regulating the conversion of precursor to proteinase is also well demonstrated in Table II and the Figure, in which immunizing activity is seen to be closely correlated with the O/R potentials of the culture of vaccines during growth. Apart from the incorporation of reducing substances in the medium, the O/R potential of static cultures may also be influenced by the presence of various naturally occurring oxygen-carrying or reducing substances (Hewitt, 1950) about which little is known, and even by the time interval between autoclaving and inoculation. Conversion of precursor to proteinase is also influenced by pH, being retarded on the alkaline side of neutrality (Table VI). It is probable also that various substances such as metals may retard or accelerate the autocatalytic process, and may be responsible for differences in the rate of conversion of precursor into proteinase noted in culture filtrates prepared from different batches of medium under the same pH and O/R conditions, and having a similar milk-clotting titre after activation. It is noteworthy that the colour of the deposited cells was greyish or black in those batches of medium in which conversion of precursor into proteinase was rapid, and which therefore yielded poorly immunizing vaccines. The reason for this is unknown, but it may be associated with the presence of such substances.

The influence of O/R potential and pH on the activity of proteinase has also been studied. A low O/R potential such as is produced by the addition of Na thioglycollate to milk was shown by Elliott and Dole (1947), and is also seen in

Table VI, to be necessary for full milk-clotting activity. Similar conditions produced in cultures of vaccines resulted in decreased immunizing activity (Table II), and this may well have been due to enhanced activity of proteinase as well as to its more rapid conversion from precursor.

Table V shows that the milk-clotting activity of filtrates is most marked at pH 8·0, rather less at pH 6·0 and considerably less at pH 7·0. The reason for the latter observation is not clear. It may be that two enzyme systems with different optimal pH are concerned in the reaction. On the other hand, destruction of the immunizing antigen was brought about to an approximately similar extent at pH 6·0 and 8·0 (Table VIII), though activity at pH 8·0 was not confirmed in Table IX.

SUMMARY.

The immunizing activity of vaccines of a weakly proteinase-producing strain of *Str. pyogenes* when grown in certain batches of digest broth, varied according to the O/R potential and pH of the cultures.

Activity was reduced with low O/R potentials and acid pH, conditions which favour the production and activation of proteinase. No such relationship was demonstrated with batches of medium not favouring the production of active proteinase.

Vaccines of strongly proteinase-producing strains with one exception showed no significant immunizing activity in mice, even when grown under conditions unfavourable to the production and activation of proteinase in culture filtrates. Possible reasons for this are discussed.

Growth of weakly or non-proteinase-producing strains in the presence of active proteinase from another strain or treatment of the heat-killed vaccine with proteinase resulted in marked loss of immunogenicity.

Immunization with crude proteinase or its precursor failed to induce significant protection against strongly proteinase-producing strains.

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THE EFFECT OF HIND LIMB ISCHAEMIA ON THE PHYSIOLOGICAL ACTIVITY OF RABBIT SKIN.

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A NUMBER of studies have recently been made on the factors affecting the mitotic activity in the mouse ear epidermis (Bullough, 1952; Laws, 1952*a*, *b*; Laws and Wright, 1952). Among these has been the demonstration by Green and Bullough (1950) that the shock state, produced by either limb ischaemia or the injection of adenosine triphosphate (ATP), markedly reduced mitosis at this site. It has been suggested by these workers that "metabolites from the post-ischaemic limb and also injected ATP interfere with carbohydrate utilization, possibly indirectly by a hormonal mechanism, and thus prevent mitosis."

At the same time other workers have been studying the effect of shock on the response of the skin to external agents. For instance, Delaunay and Pages (1945) showed that in a state of shock induced by the injection of bacterial endotoxin the cellular response to the intradermal injection of dead *Myco. tuberculosis* was reduced, as was the Arthus Phenomenon. Miles and Niven (1950) have shown that shock reduces the cellular reponse and enhances the infection produced by the intradermal injection of *Staph. aureus*, *Ps. pyocyanea* and *Cl. Welchii*, and also reduces the reactivity of the skin to the intradermal injection of histamine (Miles and Miles, 1952). These effects have been attributed to the accompanying vascular disturbances (Delaunay, Pages and Cotereau, 1946; Boquet, Delaunay, Lehoult and Lebrun, 1947; Miles and Miles, 1952).

The present experiments had a double purpose, namely to study the effects of limb ischaemia on hair growth and to attempt to determine whether the changes observed were related to changes in the skin circulation or to more specific metabolic causes. The rabbit is well adapted to such a study since it has been shown (Whiteley and Ghadially, 1951; 1953) that hair replacement on the rabbit trunk is due to cyclical waves of active hair growth on the dorsum and flanks, and that in these areas of growth mitotic activity is increased in deeper parts of the follicles (Whiteley and Ghadially, 1952). Mitotic activity in the epidermis is maintained at a low constant rate and does not show the cyclical variations seen in the mouse ear (Bullough, 1952). We have recently shown (Whiteley, Stoner and Threlfall, 1953) that the areas of hair growth in the rabbit take up more ^{32}P after its intravenous injection than do the quiescent zones, partly because of their greater extra-cellular space and partly because of their greater metabolic activity.

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METHODS.

Eighty-two Agouti and Chinchilla rabbits were used, and the methods employed were those described previously (Whiteley, *et al.*, 1953) with the following additions. Four-hour periods of ischaemia to both hind limbs were produced with tourniquets as described by Bywaters and Popják (1942), the method giving a 59 per cent mortality. Mitosis counts on hair follicles were performed as described by Whiteley and Ghadially (1952). The skin temperature was measured with a thermocouple held in a fold of skin until a constant reading was obtained. The systolic blood pressure in the central artery of the ear was measured with a Grant and Rothschild (1934) capsule.

The ear to skin circulation time was determined in three ways :

(1) With $^{32}PO_4'''$.

The rabbit was covered with a lead shield in which an aperture had been cut to allow a large fold of skin from the dorsum and left flank to be picked up and held in front of two end-window Geiger Müller tubes (G.E.C. E.H.M.2, window 2·3 mg./cm.^{2}) (Fig. 1), which were each connected to a radiation monitor (Type 1021) and the impulses from these were fed in to a double beam cathode ray oscilloscope (Cossor). The deflections of the beams were recorded photographically on Kodak recording paper (P.R. 30) travelling at 6·25 cm./sec. An intravenous needle connected by a 3 cm. length of rubber tubing to a 5·0 ml. syringe, the whole filled with 0·9 per cent NaCl, was inserted into the marginal vein of the ear. Five microcuries ^{32}P per kg. body wt., in the form of the solution described by Harrison and Raymond (1951), were injected (approx. 0·05 ml. per kg. body wt.) through a fine needle into the rubber tubing. During this time the vein proximal to the intravenous needle was occluded between the finger and thumb of an assistant. This was then released and the isotope solution rapidly washed into the vein with 1·5 ml. 0·9 per cent NaCl from the 5·0 ml. syringe. Just before the injection the camera motor was started and the shutter was opened at the moment of injection. Recordings were taken until after the initial rise in counts and then at intervals up to 10 min.

(2) *With ^{32}P-tagged erythrocytes.*

Cross-matched rabbit erythrocytes were tagged with ^{32}P as described by Reeve and Veall (1949); 1·0 ml. of the suspended cells per kg. body wt. was injected directly into the marginal ear vein and the ear-skin circulation determined as above.

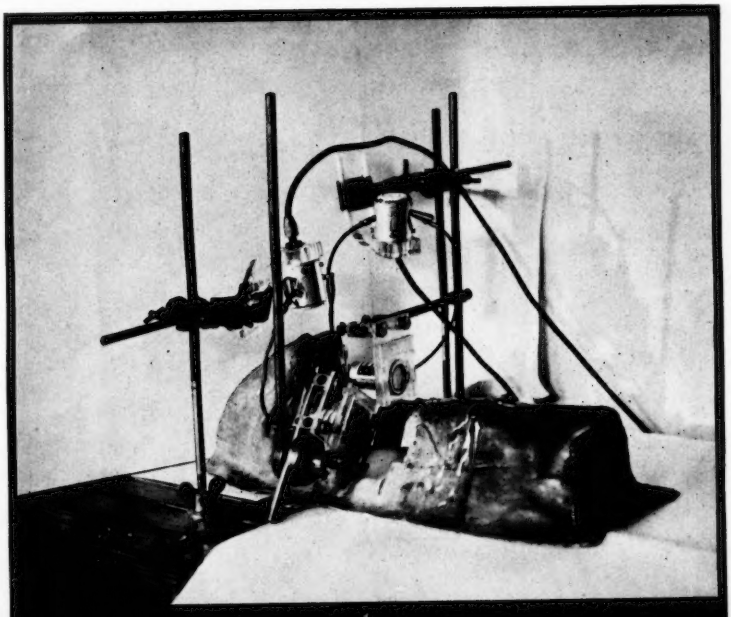
EXPLANATION OF PLATES.

FIG. 1.—Apparatus used in the determination of ear-skin circulation time showing the lead shield with the aperture through which the fold of skin is drawn and the position of the two end window Geiger Müller tubes in their " Perspex " mounts.

FIG. 2.—The skin and the autoradiograph as a mirror image from a rabbit given 75 μc. ^{32}P per kg. body wt. 1 hr. after the release of a 4 hr. period of bilateral hind limb ischaemia, the animal being killed after a further hour. There is no difference in density between the two zones and the blood vessels are visible as positive shadows.

FIG. 3.—The skin (A), the autoradiograph (B) and the fluorescence (C) from rabbit 5/53. The rabbit was killed 1 hr. after the injection of 75 μc. ^{32}P/kg. body wt. given 3 hr. after the release of a 4 hr. period of bilateral hind limb ischaemia. The Na fluoresceinate (0·15 ml./kg. body wt.) was given 20 min. before death. It will be seen that there is good autoradiographic differentiation and resolution between the skin showing active hair growth (dark area in A) and the quiescent skin on the left side, and that there is considerable fluorescence (light areas) from that area. On the other side there is poor autoradiographic resolution and differentiation between the two zones and there is no fluorescence. This difference in response between the two sides is sharply demarcated at the mid dorsum (↓). The fluorescence was photographed by reflected light from a Hanovia chromatolite on Pan F film through a Wratten 58 B filter, exposure 10 min. f.2.8.

	Left side.	Right side.
Radioactivity ratios	2·45	1·26
Density ratios	3·0	1·35



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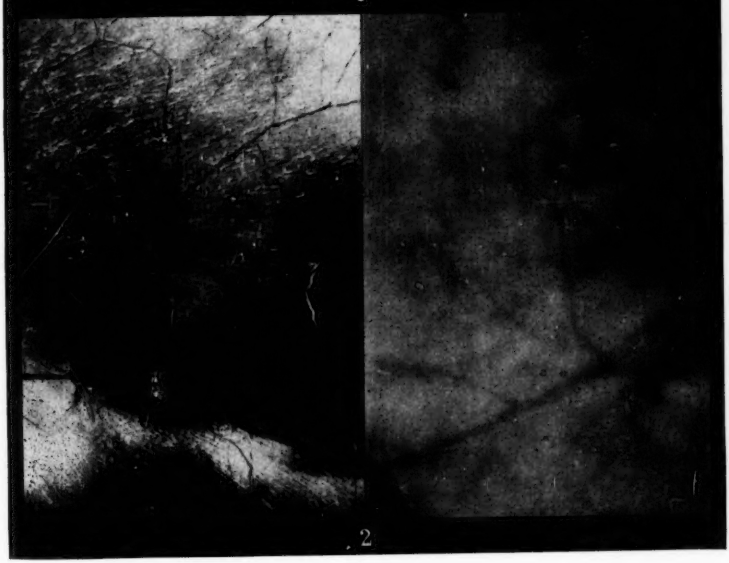

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Whiteley, Stoner and Threlfall.

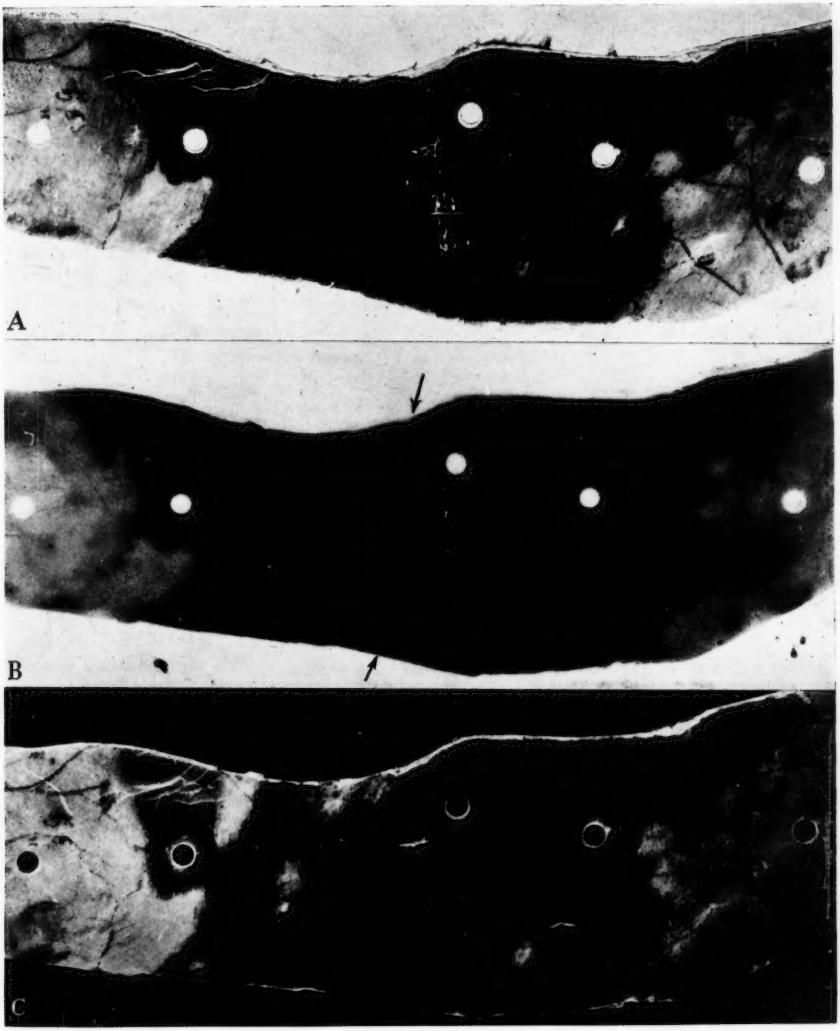

Fig. 3.

(3) *With Na fluoresceinate.*

A 20 per cent solution of Na fluoresceinate (Macgregor and Wayne, 1951) was injected directly into the marginal vein of the ear in a dose of 0·15 ml. per kg. body wt. and the time to the first appearance of fluorescence in the skin, viewed in the light from a Hanovia "chromatolite," determined with a stop watch.

RESULTS.

Effect of Hind Limb Ischaemia on the Mitotic Activity of the Follicles.

Serial biopsies from 4 rabbits subjected to 4 hr. hind limb ischaemia showed a rapid and profound fall in the mitotic activity in the deeper layers of the follicles which started during the period of ischaemia. This latter finding is contrary to that observed by Green and Bullough (1950) in mouse ear epidermis, where the depression in mitotic activity did not begin until after the release of the tourniquets. It will be seen from Table I that in the rabbit which recovered the mitotic rate, which was lowest at 6 p.m. had returned to normal at 24 hr., but in the two that died after 6 p.m. the mitotic count remained at the low level. In 4 other rabbits, killed at 6 p.m., the fall in mitotic rate had been of a similar order. This suggests that the mitotic rate is lowest about 4 hr. after the removal of the tourniquets, and that its subsequent behaviour depends on the clinical state of the animal.

Numerous aggregates of clumped nuclear material were seen in the follicles, usually after release of the tourniquets, which were not seen in the control material. It was thought that these aggregates might possibly represent mitotic figures altered and distorted as a result of changes produced by hind limb ischaemia.

Effects of Hind Limb Ischaemia on the Uptake of ^{32}P by Rabbit Skin.

It has been shown (Whiteley *et al.*, 1953) that areas of rabbit skin in which there is active hair growth take up more ^{32}P than areas where there is no hair growth. Five minutes after the intravenous injection of the isotope the ratio (growing/quiescent) of the radioactivity of equal areas of skin was 1·8 and the ratio of the photographic densities of the autoradiographs was 1·5–1·65. The areas of active hair growth were accurately depicted as dense shadows on the autoradiographs. After one hour there was even greater concentration of the isotope in the areas of hair growth, the ratio of the radioactivity of equal areas of skin being 2·3 and that of the photographic density 2·4 (range 2·0–2·8). This difference between the two types of skin was still more apparent after 24 hr.

This picture was considerably modified by limb ischaemia. In 6 animals, killed 5 min. after the intravenous injection of 75μc. ^{32}P per kg. body wt. 3 hr. 55 min. after a 4 hr. period of ischaemia of both hind limbs, considerable variation was found in the relative amounts of isotope in the growing and quiescent skin. The ratio (growing/quiescent) of radioactivity varied from 0·54–2·1 (mean 1·7). The autoradiographs showed a similar variability, the ratio of the densities ranging from 0·59–2·1 (mean 1·36). All the autoradiographs showed poor resolution between the two zones even with a density ratio of 2·1, and in one animal with a dorsal zone of hair growth the normal ratio was reversed. The major blood vessels were visible as positive shadows in all six autoradiographs. This was only very occasionally seen in any of the autoradiographs from normal animals. The

TABLE I.—*Effect of Bilateral Limb Ischaemia on the Mitosis Rate in Hair Follicles in Zones of Hair Growth of Rabbit Skin.*

	Number of mitoses per 100 follicles.														
Time	9.30. a.m.	10.0.	11.0.	12.0 noon.	2.0.	3.0.	4.0.	5.0.	6.0.	8.0.	10.0.	0.0 a.m.	6.0.	10.0.	2.0. p.m.
Control rabbit . .	142	—	—	—	—	196	—	—	174	—	150	—	—	136	—
„ „ . .	111	—	—	107	152	163	—	—	168	160	142	—	—	184	216
„ „ . .	112	—	—	—	160	138	—	—	120	184	192	—	—	176	—
„ „ . .	143	—	—	—	—	—	—	—	169	—	—	—	—	—	—
	Period of ischaemia.														
Experimental rabbit .	137	—	—	—	86	75	34	43	16	12	17	9	Died	—	—
„ „ .	79	—	—	51	68	52	67	66	24	30	32	—	95	81	104*
„ „ .	—	—	—	69	47	26	21	17	24	Died	—	—	—	—	—
„ „ .	152	—	—	146	120	68	73	37	32	45	29	14	Died	—	—
„ „ .	119	—	—	—	—	—	—	—	22*	—	—	—	—	—	—
„ „ .	88	—	—	—	—	—	—	—	19*	—	—	—	—	—	—
„ „ .	149	—	—	93*	—	—	—	—	—	—	—	—	—	—	—
„ „ .	—	—	—	—	—	—	—	—	32*	—	—	—	—	—	—
„ „ .	—	—	—	—	—	—	—	—	17*	—	—	—	—	—	—

* = Killed.

degree of differentiation between the two zones did not appear to be in any way related to the position of the areas of hair growth on the trunk.

In 3 rabbits, killed 1 hr. after the intravenous injection of 75μc. ^{32}P per kg. body wt. given 1 hr. after a 4 hr. period of ischaemia to both hind limbs, there was little difference in the amount of isotope in the two zones, the density, as determined by autoradiography, being 1·0–1·5 (mean 1·2). In one (Fig. 2) there was no difference between the two zones and the blood vessels were clearly visible. In 5 rabbits killed 4 hr. after a similar period of ischaemia, the ^{32}P being given 1 hr. before death, a similar picture was seen. Here the ratio of radioactivity was 0·9–1·56 (mean 1·17) and the ratio of density 0·7–1·3 (mean 1·08); there was little or no resolution between zones, and blood vessels were visible as positive shadows in 4 rabbits. In a further animal with a dorsal hair growth zone the findings were rather more complex (Fig. 3, A and B). On one side there was good differentiation, the radioactivity ratio was 2·45 and the density ratio 3·0, while on the other side there was poor differentiation, the radioactivity and density ratios being 1·26 and 1·35 respectively. It will be observed that the difference in response between the two sides is sharply demarcated at the mid-point in the dorsum. This animal will be referred to later.

In less severely affected animals which survived for 24 hr., and in one animal killed during the period of ischaemia the normal pattern of increased radioactivity in the areas of hair growth was found.

In the normal rabbit the total electron flux from both zones of the skin is greatest about 5 min. after the ^{32}P injection because of the great radioactivity of the plasma at that time. After this point the electron flux gradually declines. After hind limb ischaemia this is altered, for the maximum flux is never as great as from normal skin and is not reached until about 1 hr. after the injection.

Blood Pressure Changes after Hind Limb Ischaemia.

Serial blood pressure readings in animals before, during and after hind limb ischaemia showed a rise during the period of ischaemia followed by a fall after release of the tourniquets (Fig. 4). Three hours after release the blood pressure, appeared to rise somewhat. This was because it could only be recorded in the least severely affected animals. In others, at this time, there was a spasm of the central ear artery making it impossible to obtain a reading in these animals as there was no pulsatile flow, the artery shutting down completely as soon as the capsule was inflated. These observations indicate a considerable disturbance in the peripheral circulation.

Effect of Limb Ischaemia on the Ear-Skin Circulation Time.

In the normal animal the $^{32}PO_4'''$ circulation time varied with the position of the hair growth zone (Table II). In the animal with no growth zones the circulation time to the flank was slightly less than to the dorsum, and the subsequent number of disintegrations per second was higher in the flank (Fig. 5, R.115) whereas with a dorsal growth zone (Fig. 5, R.112) the findings were reversed. With tagged cells the circulation time in the normal rabbit was longer (Table II, Fig. 6, R.131) and there was no clear relationship between the circulation times to the dorsum and flank and the presence or absence of growth over the dorsum. The time for the complete mixing of the tagged cells varied but did not exceed

10 min. Fluorescein circulation times (Table II) were of a similar order and agreed well with those determined in the rabbit by Boquet *et al.* (1947).

In the post-ischaemic animal the $^{32}PO_4'''$ circulation time was prolonged and the subsequent rapid rise in the number of disintegrations was not seen (Fig. 5,

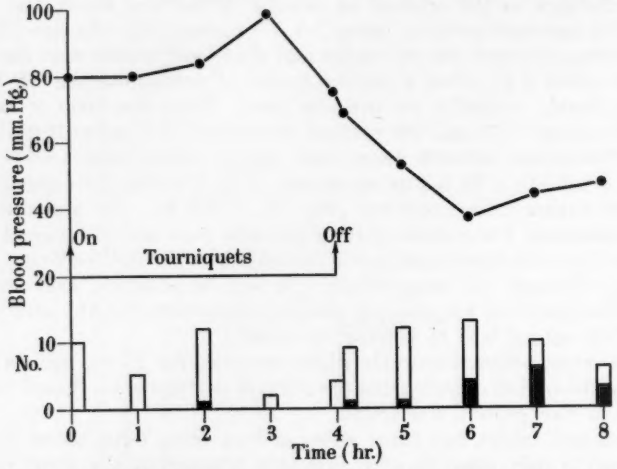

FIG. 4.—Changes in systolic blood pressure, as measured in the central artery of the rabbit's ear, during and after the application of tourniquets to both hind limbs. The number of animals observed at each time is shown by the height of the columns in the histogram at the bottom of the figure. The blocked-in portions of the columns show the number in which a reading could not be obtained because of arterial spasm. The points on the curve are the means of the values obtained.

TABLE II.—*Circulation Time (Seconds) from Ear to Flank and Dorsum in Control and Tourniquet Animals With and Without Dorsal Zones of Hair Growth.*

	Control rabbits.				Rabbits 2·5–4·0 hr. after 4 hr. period of bilateral hind limb ischaemia.			
	No growth zone.		Growth zone on dorsum.		No growth zone.		Growth zone on dorsum.	
	Dorsum.	Flank.	Dorsum.	Flank.	Dorsum.	Flank.	Dorsum.	Flank.
^{32}P solution (5 μc. per kg. (body wt.)	—	—	4·5	—	—	—	—	—
	4·2	3·4	4·9	—	10·3	10·1	n.d.	11·0*
	7·0	5·3	6·2	—	13·0	13·0	n.d.	17·0*
	4·5	4·3	3·1	3·4	7·8	8·2	11·0	11·0
	5·3	4·4	4·4	5·1	—	—	—	—
^{32}P-tagged R.B.C. (1·0 ml. per kg. body wt.)	6·0	8·0	9·0	8·0	—	—	20·0	19·0*
	6·0	10·0	6·0	7·0	—	—	18·0	20·0
	7·0	8·0	—	—	—	—	27·0	17·0
	8·0	9·0	—	—	—	—	>50·0	6·0
	—	—	—	—	—	—	47·0	44·0*
20 per cent sodium fluoresceinate (0·15 ml. per kg. body wt.)	—	9·0	—	—	—	—	—	15*
	—	7·0	—	—	—	—	—	128
	—	6·0	—	—	—	—	—	66
	—	—	—	—	—	—	—	143
	—	—	—	—	—	—	—	186

* Subsequently died. n.d. = undeterminable.

R.117, R.119). The rate of this rise was to some extent dependent on the clinical state of the animal; *e.g.*, Rabbit 119 (Fig. 5) subsequently died, and Rabbit 117 (Fig. 5) recovered. In 2 animals with dorsal growth zones which subsequently died the radioactivity of the dorsal skin never rose above background.

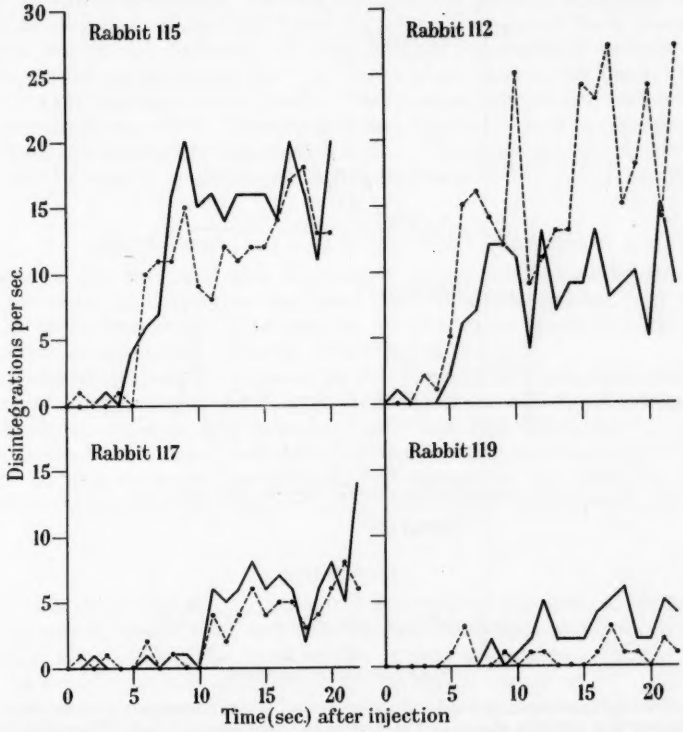

FIG. 5.—The radioactivity of the skin of the flank (———) and dorsum (- - - -) as recorded by the end window counters shown in Fig. 1 after the injection of 5 μc. ^{32}P/kg. body wt. into the marginal vein of the ear.

Rabbit 115.—Control. No hair growth zone.
Rabbit 112.—Control. Dorsal hair growth zone.
Rabbit 117.—4 hr. period of ischaemia, ^{32}P given 3 hr. 15 min, after release of the tourniquets. No hair growth zone. Animal survived.
Rabbit 119.—4 hr. period of ischaemia, ^{32}P given 3 hr. 30 min. after release of the tourniquets. Dorsal hair growth zone. Animal died overnight.

This prolongation of the circulation time was also demonstrated with tagged cells. The effect on the ear-dorsum time was greater than on the ear-flank time even though there was a dorsal growth zone (Fig. 6, R.122). A relationship was again seen between the clinical state and the subsequent rise in the number of disintegrations (Fig. 6). This rise was always greater in the flank than the dorsum. Both findings were thought to be related to the presence in the flank of the large blood vessels which supply the skin of the trunk. In many cases the mixing

time was delayed as found by Delorme, Mukherjee and Rowlands (1952) in haemorrhagic shock in the dog.

The fluorescein circulation times were much longer because of the extreme difficulty in determining the end point. Fluorescence was usually seen first in the flanks. Even after 20 min. there were large areas of skin, usually dorsal, which

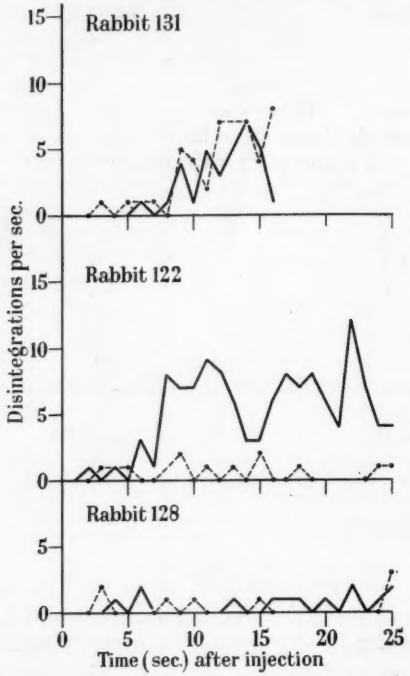

FIG. 6.—The radioactivity of the skin of the flank (———) and dorsum (- - - -) as recorded by the end window counters shown in Fig. 1 after the injection of 1·0 ml. ^{32}P tagged erythrocytes/kg. body wt. into the marginal vein of the ear of rabbits with a dorsal hair growth zone.

Rabbit 131.—Control.

Rabbit 122.—4 hr. period of ischaemia ; the tagged cells were given 3 hr. after release of the tourniquets. Animal survived.

Rabbit. 128.—4 hr. period of ischaemia, the tagged cells were given 3 hr. 15 min. after release of the tourniquets and the animal died 1 hr. later.

did not fluoresce. This patchy fluorescence in the skin was also observed by Boquet *et al.* (1947) in endotoxin shock in rabbits where it was associated with a prolonged ear-eye circulation time. The rabbit in Fig. 3, already referred to, received fluorescein, and examination of the dried skin (Fig. 3, C) showed that it only fluoresced on one side ; this was the side with high radioactivity and density ratios between the growing and quiescent zones ; on the other side there was no fluorescence, low ratios and poor autoradiographic differentiation. It seems reasonable to suppose that this difference in the response of the two sides could be explained by unilateral vascular spasm.

Effect of Limb Ischaemia on Skin Temperature.

There was a fall of about 3°(C.) in the skin temperature from a normal of 37° 2 hr. after the application of the tourniquets in a group of 4 rabbits, after which the skin temperature was maintained at the new level even after removal of the tourniquets. In two animals, with no zones of hair growth, subjected to a 4 hr. period of ischaemia to both hind limbs, in which dorsal and flank temperatures were taken, the initial difference of 0·25° became progressively greater, until at 6 p.m. the dorsal temperature was 2·25° lower than that in the flank. In order to determine the maximal rate of cooling, two normal rabbits were killed and their skin temperatures recorded. There was a rapid fall of 8° in 3 hr. and no increase in the difference between the dorsum and flank. The difference seen in the shocked animal may be due to a disproportionate decrease in the blood supply to the dorsum.

Blood Content of the Skin after Limb Ischaemia.

In view of the disturbed skin circulation in the post-ischaemic state it was thought necessary to determine the blood content of the growing and quiescent zones. In both control and experimental animals more blood was found in the growing areas as previously reported (Whiteley, *et al.*, 1953).

The means of the amount of blood in the growing and quiescent zones in the experimental animals were 0·55 and 0·24 mg. blood per sq. cm. skin respectively, and in the control group they were 0·71 and 0·32 mg. blood per sq. cm. skin. The difference of the mean values between the growing and quiescent zones was significant in both the experimental and control animals, but there was no significant difference between the means of comparable areas of skin in the two groups.

DISCUSSION.

Our results show that the physiological activities of the skin, as judged by the mitotic activity of the growing hair follicles and the uptake of ^{32}P by the areas of hair growth, are considerably disturbed by muscle ischaemia ; there is lowering of follicular mitotic activity and failure of differential uptake of ^{32}P. The extent of these disturbances in the normal behaviour of rabbit skin is related to the clinical state of the animal, becoming apparent during the period of ischaemia, reaching a maximum about 4 hr. after release of the tourniquet and, if the animal survives, returning to normal after 24 hr. Our previous experiments on ^{32}P uptake by rabbit skin (Whiteley *et al.*, 1953) showed a marked uniformity of behaviour both from animal to animal and within the same animal. This was lost after limb ischaemia, when there was a wide variation in the degree of differential uptake both from rabbit to rabbit and in different parts of the skin of the same rabbit.

In considering the possible mechanisms involved it was essential to determine first the availability of metabolites, including ^{32}P, to the active cells. This had become clear from similar work on the effect of shock on the uptake of ^{32}P by rat muscle (Stoner and Threlfall, unpublished work) and was reinforced, from a different angle, by the views of Miles and the French workers mentioned above. The circulation through the skin after muscle ischaemia was therefore investigated and found to be considerably altered. Not only was the ^{32}P ear-skin circulation time prolonged but the subsequent rise in radioactivity was much less than in the normal animal. In some rabbits there was no entry of isotope into the

dorsal zone during the first 60 sec. after injection. These observations were confirmed with tagged cells. These results considered in conjunction with the blood content of the skin determined by the method of Lowry and Hastings (1942) provided definite evidence of the stagnation of blood in the skin after limb ischaemia. Since phosphorus is ultilized by the cells an inert substance was also used in these experiments. Fluorescein was chosen and gave similar results. It was also observed that the initial fluorescence in the shocked animal appeared in the flank in relation to the major blood vessels and that, even after 20 min., areas of skin, usually on the dorsum, did not show any flourescence. These vascular changes in the skin can be most easily attributed to local vascular spasm and this view is supported by the frequency with which spasm was seen in the central artery of the ear after hind-limb ischaemia and the increasing temperature difference between the flank and the dorsum.

Clearly these vascular changes in the skin coupled with the fall in systolic blood pressure will reduce the supply of isotope to the skin. It has been shown by Johnson, Cavert and Lifson (1952) that the velocity of blood flow is an important factor in determining the rate of uptake of an isotope by a tissue and it would seem that the poor differential uptake of ^{32}P by the different zones of the rabbit skin after limb ischaemia is related to its reduced availability. This would seem to be the most satisfactory explanation of the appearance seen in Fig. 3. Here, on the side where ^{32}P was available, as judged by fluorescence, the normal pattern of uptake was seen, while on the other side, where there was poor availability as judged by the absence of fluorescence, the normal pattern was abolished.

If the supply of ^{32}P to the skin is reduced in this way by hind limb ischaemia then that of O_2 and other essential metabolites must also be reduced. In the face of this it would seem unnecessary to seek further for an explanation of the fall in mitotic rate. It is relevant to mention here that systemic cortisone, postulated as an intermediary agent in the depression of epidermal mitosis in the mouse ear after hind limb ischaemia (Green and Ghadially, 1951; Green and Savigear, 1951) did not have any effect on the mitotic activity in the follicles or on the uptake of ^{32}P by the growing zones of rabbit skin.

When these results are considered in conjunction with those of other workers it would appear that the changes produced by shock in both the normal behaviour of the skin and its responses to external stimuli are due to the accompanying vascular changes.

The experiments described in this and the previous paper illustrate several of the difficulties encountered in the study of skin reactions in the rabbit. Firstly, attention must be paid to the distribution of the hair growth zones since their blood supply and metabolic behaviour is so different from that of the quiescent areas. This makes it difficult to do experiments on albino rabbits. Secondly, a skin reaction cannot be said to be adversely affected by a general bodily condition unless it can be shown that adequate metabolic requirements are still being supplied to the skin.

SUMMARY.

The effect of hind limb ischaemia on the physiological activities of rabbit skin has been investigated by studying its effect on the mitosis rate in the growing hair follicles and on the uptake of ^{32}P by the different areas of skin.

It was found that hind limb ischaemia depressed the mitotic rate of these cells and interfered with the normal pattern of ^{32}P uptake.

These effects are attributed to the alterations in the skin circulation which follow hind limb ischaemia.

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THE TUBERCULIN REACTION OF THE CEREBRAL MENINGES IN THE GUINEA-PIG.

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TUBERCULIN sensitivity follows the establishment of a tuberculous infection; this may be shown in the skin by a Mantoux test and is seen in the lungs during an attack of so-called epituberculosis. It was once accepted that the meninges shared in the general sensitivity of the body, but of recent years there seems to have grown up an impression that the central nervous system and its coverings are incapable of allergic responses. The object of the work reported here was to investigate the response of the leptomeninges to tuberculo-protein in normal and in sensitised animals, and to compare the histological picture with the tuberculin reaction as seen in the skin.

The general plan of the experiments followed the usual method of investigating sensitivity reactions. Animals which were known to be skin-sensitive were given an injection of tuberculo-protein locally into the tissue under investigation, in this case into the subarachnoid space via the cisterna magna. The tuberculo-protein used was the Weybridge Purified Protein Derivative, or P.P.D. (Green, 1946).

METHODS

The animals chosen for study were guinea-pigs because they become skin-sensitive to tuberculin quickly and certainly, and are susceptible to human strains of *Myco. tuberculosis*, in which we were especially interested.

Primary sensitisation.

One of three antigens was used: Living human tubercle bacilli (H37Rv); a heat-killed preparation of the same human strain; and living murine tubercle bacilli (vole bacilli, O.V.). In each case 1·0 ml. of a heavy suspension in saline was injected into the groin; no attempt was made to calibrate the dose as the object was simply to render the animal skin-sensitive. It soon became apparent that the meningeal reactions were independent of the antigen used, and the results are therefore treated together. Most animals received one sensitizing injection, but a few had two.

Sensitivity tests in skin.

Animals were skin-tested by an intradermal injection of 0·2 ml. Old Tuberculin 1:10, or purified protein derivative (P.P.D.), at intervals after the sensitising inoculation and were positive at the time of the intracisternal injection. The skin tests were read after 48 hr. and the usual nodule of induration with reddening of the skin, with or without necrosis, was accepted as a positive reaction. Animals with a positive skin reaction we refer to throughout as "sensitised" animals.

Sensitivity tests in meninges.

Under light ether or nembutal anaesthesia 0·2 ml. (1·5 μg.) P.P.D. solution in saline was injected into the cisterna magna of sensitised guinea-pigs (Table I*a*), and the animals

were killed with chloroform or nembutal after intervals of from 8–48 hr. All animals, including those with progressive tuberculosis, appeared healthy and were active at the time of the intracisternal injection. The animals were killed with an overdose of anæsthetic. The brains were removed immediately and fixed in a mixture of 10 per cent formalin in 60 per cent alcohol for several days before being cut coronally into blocks, embedded in paraffin, and sectioned. The sections were stained with Ehrlich's acid haematoxylin and eosin or Weigert's iron haematoxylin and Van Gieson.

Controls.

As controls, non-sensitised animals were given intracisternal injections of either P.P.D. (Table 1*b*) or saline in order to study the reaction of the non-sensitised meninges. Saline was also injected intracisternally into two sensitised animals. The control animals were injected at the same time and the volume of fluid given was the same as in the main group of experimental guinea-pigs ; they were killed 24 hr. later, as this was found to be the time when there was a good reaction in sensitised animals.

RESULTS.

Both the experimental animals and the controls sometimes had a fit immediately after the intracisternal injection, but, excluding one or two which died immediately as a result of damage to the medulla and are not included in the series, they recovered within an hour.

Macroscopically there was no visible exudate, and the brain and meninges appeared normal in all animals, apart from occasional needle-marks in the medulla. Histologically, however, the 15 sensitised guinea-pigs (Table 1*a*) which had received intrathecal P.P.D. all showed a definite cellular exudate in the leptomeninges. This was significantly greater than in the 9 non-sensitised control animals (Table 1*b*). Thus, the response of the sensitised leptomeninges differs markedly from the response in non-sensitised animals ; that this difference is due to an allergic reaction, and is not only a non-specific increase in the irritability of tuberculous animals*, was shown by the lack of comparable exudate in the two sensitised animals which were given intracisternal saline in place of P.P.D.

The amount of this exudate (and by exudate we refer to an excess of cells, in the subarachnoid space, not an outpouring of fluid) 24 hr. after intracisternal P.P.D. is illustrated in Fig. 1, while the effect of intracisternal P.P.D. on a normal non-sensitised control animal is shown in Fig. 2. The photomicrographs are of coronal sections through the floor of the third ventricle and basal meninges with the choroidal fissure passing obliquely across the field. In the sensitised animal (Fig. 1) the whole subarachnoid space of the basal cistern and choroidal fissure is filled with cells (exudate), whereas in the non-sensitised and intact animal (Fig. 2) there are comparatively few cells. The difference is particularly striking along the choroidal fissure.

Figs. 3 and 4 show the region of the basilar artery 24 hr. after intracisternal P.P.D. in a sensitised and a non-sensitised animal respectively. In the sensitised guinea-pig there is a massive exudate and perivascular cuffing of the penetrating vessels.

The distribution of the exudate was interesting, as in all cases it was maximal at the base of the brain (the characteristic site of exudate in human tuberculous meningitis). In the more intense reactions it extended around the brain stem

* Several authors have noted that the response to non-specific irritants is greater in tuberculous than in non-tuberculous animals ; see Dienes and Mallory (1932) ; Laporte (1934) ; Lurie (1939).

TABLE 1.—*Intrathecal Injection of P.P.D. and Saline into Sensitised and Non-Sensitised Guinea-Pigs.*

G.P. No.	Antigen and skin sensitivity.	Intracisternal Injection of P.P.D. 7·5 μg. per ml.	Survival Time in hours.	Meningeal Exudate.	Type of Cell in Exudate.
		Table Ia.			
3	+ (H37Rv)	0·2 ml.	24	+++	M=P
6	+ (O.V.)	0·2 „	24	+++	M>P
9	+ (killed culture)	0·2 „	24	+++	M>P
4	+ (H37Rv)	0·1 „	24	++++	M=P
7	+ (O.V.)	0·1 „	24	++	M>P
8	+ „	0·1 „	24	++	M>P
10	+ (killed culture)	0·1 „	24	++	M>P
11	+ „ „	0·1 „	24	++	M>P
76	+ (O.V.)	0·2 „	24	+	M>P
46	+ „	0·2 „	8·5	+	M
5	+ (H37Rv)	0·1 „	11	++++	P>M
48	+ (O.V.)	0·2 „	16	++	M>P
49	+ „	0·2 „	48	+	M>P
50	± „	0·2 „	48	+	M
52	+ „	0·2 „	48	+	M
		Table Ib.			
13	— —	0·2 ml.	24	—	—
14	— —	0·2 „	24	—	—
15	— —	0·2 „	24	—	—
16	— —	0·2 „	24	—	—
30	— —	0·2 „	24	±	Some haemorrhage
81	— —	0·2 „	24	±	—
82	— —	0·2 „	24	±	Occ. M & P
83	— —	0·2 „	24	±	—
79	— —	0·2 „	27	—	—

O.V. *Myco. tuberculosis* murine (Vole bacillus living).
H37Rv *Myco. tuberculosis* (human, living culture).
Killed culture Heat-killed culture H37Rv.
M Mononuclears.
P Polymorphonuclears.

and cerebellum (Fig. 5), but only in the most intense reactions was any significant amount of exudate found over the cerebral hemispheres. The distribution of the exudate appeared to us to depend on the severity of the reaction, being simply more or less widespread, and not to show a variation related to the duration of the reaction as was reported by Burn and Finley (1932); nor did it show any relationship to the tuberculous state of the guinea-pig.

The type of cell found in the exudate varied, but in general the reaction was predominantly mononuclear, although polymorphonuclear cells were present in most cases and occasionally (No. 3 and 4) polymorphonuclears were as frequent as the mononuclears (Fig. 6), while in one case (No. 5) they actually predominated. At 48 hr. the exudate was mainly mononuclear; some cells in the exudate had pyknotic nuclei and some of the macrophages had ingested polymorphonuclears (Fig. 7). The animal killed at 8½ hr. had a slight purely mononuclear exudate (Fig. 8).

No necrosis of the meninges comparable with the epithelial destruction in the skin was present nor was any arteritis or gross damage to the brain tissue found.

Further Experiments.

Intracisternal injection of tubercle bacilli.

A few animals were given tubercle bacilli intrathecally. Virulent human bacilli (H37Rv), either the living organisms or a heat-killed suspension of the same culture, were injected intracisternally into sensitised and non-sensitised guinea-pigs. One animal of each set was killed after 24 hr. (Table II). All four animals showed a definite cellular exudate of a mixed polymorphonuclear and mononuclear type. The amount of exudate was perhaps greater in the sensitised than in the non-sensitised animals, but without further experiments it is impossible to say whether the difference was significant.

TABLE II.—*Intrathecal Injection of Myco. Tuberculosis into Sensitised and Non-Sensitised Guinea-Pigs.*

G.P. No.	Sensitising Antigen.	Skin Sensitivity.	Intrathecal Injection of *Myco. tuberculosis* (human).	Survival Time in days.	Meningeal Exudate.
A. *Living Organisms Intrathecally.*					
51	Living vole bacilli	+	0·2 ml.	1 (well)	Much
39	Killed culture (human)	+	0·2 „	21 (sick)	„
41	„ „ „	+	0·2 „	31 „	Massive
56	*Nil*	—	0·2 „	1 (well)	Some
19	„	—	0·1 „	10 (moribund)	Massive
23	„	—	0·1 „	11 „	„
20	„	—	0·1 „	12 „	„
22	„	—	0·1 „	12 „	„
21	„	—	0·1 „	14 (died)	„
B. *Killed Culture Intrathecally.*					
45	Killed culture (human)	+	0·2 „	1 (weak)	Massive
35	„ „ „	+	0·2 „	95 (well)	Considerable
34	„ „ „	+	0·2 „	367 „	Slight but definite
55	*Nil*	—	0·2 „	1 (well)	Considerable

Seven animals, 2 sensitised and 5 non-sensitised, which had been given living virulent bacilli intrathecally were left until moribund. Of these, the 5 non-sensitised animals died in 10–14 days, while the 2 sensitised ones survived for 21 days and 31 days respectively (Table IIA). All had a massive meningeal exudate, in which in some cases foci of early caseation were found. It is interesting that the two sensitised guinea-pigs which received 0·2 ml. killed culture intracisternally, and clinically never appeared ill, showed a moderate but definite exudate 3 months and one year respectively, after the injection (Table IIB).

Intraperitoneal injection of tuberculo-protein.

Three sensitised guinea-pigs were given enough tuberculo-protein intraperitoneally to kill them. One died in 6 hr., one between 9 and 18 hr. and one between 36 and 42 hr. (these last two were found dead in the cage). In these three animals there was marked congestion not only of the surface (meningeal) vessels, but also of those in the substance of the brain ; this was accompanied by some subarachnoid blood and a very little mononuclear exudate, which was in no way comparable with the intense exudate produced by intracisternal P.P.D.

DISCUSSION.

These experiments show that P.P.D. introduced intrathecally into sensitised guinea-pigs produces a tuberculin reaction in the leptomeninges showing similarities to the Mantoux reaction in the skin. The allergic nature of this reaction is shown by the fact that it is obtained only in animals which have a positive skin reaction and that a non-specific substance (saline) does not elicit it. It resembles the tuberculin reaction in the skin both in being mainly an infiltration of cells and in developing after some hours, whereas the anaphylactic type of reaction is essentially an outpouring of fluid occurring extremely rapidly.

Considerable evidence has already accumulated to show that tuberculin sensitivity is not confined to the skin. It has been demonstrated experimentally in the testis (Long, 1924), the kidney (Long, Huggins and Vorwald, 1930), and the lungs (Austrian and Willis, 1926). It might therefore be supposed that the leptomeninges also would become sensitised with the other tissues and indeed Rich (1944) considers that this is a major factor in human tuberculous meningitis. Experimental evidence of such a reaction has not been lacking : Borrell's classical observation of the tuberculin reaction in 1900 followed the intracerebral inoculation of tuberculin ; Calmette (1922) accepted that " the brain " became sensitised with the other tissues ; and more recently the histological studies of Burn and Finley (1932) have demonstrated a meningeal exudate after the intracisternal injection of old tuberculin in tuberculous guinea-pigs. Smith and Vollum (1950) have shown that there is an increase of cells in the cerebrospinal fluid in Mantoux-positive humans, within 24 hours of an intrathecal injection of P.P.D.

However, in spite of this evidence the meningeal reaction has tended to be overlooked and even denied. Somerfeld and Ziskind (1931) failed to find any evidence of a specific reaction after the intracisternal injection of tuberculin into tuberculous and non-tuberculous guinea-pigs. Their conclusion was, however, reached after a macroscopic examination only, and this is valueless, as quite a massive microscopic exudate is often not apparent to the naked eye. Also, Somerfeld and Ziskind used as non-specific control substances ones which were themselves toxic. Bickford (1932) who injected a number of substances including tuberculin into the cistern of rabbits, was also not impressed with the difference in reaction between the tuberculous and the uninfected animals. However, it is well known that rabbits do not readily develop tuberculin sensitivity and in any case he was using such large doses of old tuberculin (2·0 mg.) that the substance itself may well have caused a direct chemical irritation, masking any allergic response.

The meningeal response to tuberculin is similar to the skin reaction in that the most striking feature of both is a local accumulation of cells. The type of cell present is also the same in both tissues, being a mixture of polymorphonuclears and mononuclears. All workers are agreed that both these types of cell are found in the skin lesions, and this we have confirmed on our own material. Dienes and Mallory (1932), using guinea-pigs, thought, on evidence that is not altogether convincing, that the tuberculin reaction was essentially one of large mononuclear cells. In skin lesions they found over 40 per cent polymorphonuclears by 6 hours and 60–70 per cent at 24 hours, but this they considered secondary to necrosis of the epithelium. They appear to have based their conclusions largely on the finding that mononuclears only were present at 2 hours. Gell and Hinde (1951), who have studied the skin reaction in rabbits at 4 and 22 hours, report the presence

of polymorphonuclears without necrosis. They found only up to 40 per cent polymorphonuclears in tuberculin reactions, whereas in a non-specific inflammatory reaction over 50 per cent of the cells were polymorphonuclear. In the meninges as in the skin we found that mononuclear cells formed the chief part of the exudate, although polymorphonuclear cells were usually present and indeed sometimes made up a considerable part of this exudate. The presence of polymorphonuclears was not apparently due to necrosis of the meninges. No deduction can be drawn as to the early stage of the reaction, as only one animal was killed at 8 hours, but in general it seems that the meningeal response is not unlike that of skin.

The interest of these experiments in relation to the human disease is that the allergic meningeal reaction must occur in almost all cases of human tuberculous meningitis. It is extremely rare, not to say almost unknown, for tuberculous meningitis to occur as a primary infection, so that the naturally occurring disease takes place within sensitised meninges. Rich and McCordoch (1929) showed clearly that in the great majority of cases tuberculous meningitis follows the establishment of a caseous focus (Rich's focus) in or adjacent to the meninges. Tubercle bacilli enter the subarachnoid space from this focus, thus reproducing naturally a condition closely resembling that of our experiments. It is perhaps less often remembered that, as a corollary, Rich regards a large part of the clinical and pathological picture of diffuse tuberculous meningitis as a manifestation of the hypersensitivity reaction. It is not possible to tell from the results of the present experiments how much of the characteristic histological picture of the naturally occurring disease is due to hypersensitivity. We found that the introduction of either living or heat-killed tubercle bacilli into the subarachnoid space of sensitised guinea-pigs caused, within 24 hours, a reaction similar to the allergic response to P.P.D., although of course living organisms later gave rise to a progressive meningitis. On the other hand, in non-sensitised animals a comparable reaction was seen 24 hours after the intracisternal injection of tubercle bacilli (Table II). All these animals, however, were given an overwhelming dose of organisms, much in excess of most spontaneous infections; this may possibly have masked a difference in reaction between the sensitised and non-sensitised animals. Necrotising arteritis, a common finding in human tuberculous meningitis, was not seen in the guinea-pigs either in P.P.D. reactions or in animals with progressive meningitis.

It is commonly thought that Mantoux-positive (sensitised) patients have a higher resistance than Mantoux-negative ones and in this connection it is interesting that we were able to confirm though on a very small number of animals, Soper and Dworski's report (1930) that sensitised animals had a greater resistance to subarachnoid infection than non-sensitised ones.

When the tuberculo-protein is not injected locally (intracisternally) there is little, if any, meningeal response, which is what one would expect since the tuberculin reaction is essentially a local phenomenon being presumably dependent on the quantity of antigen present. Burn and Finley (1932) noted that injections of tuberculin into the carotid artery were not followed by a meningeal exudate. We have found that after intraperitoneal injections of tuberculo-protein there was very little, if any, meningeal exudate; but that there was a marked congestion of both the surface vessels and those in the substance of the brain. A slight increase of cells in the subarachnoid space was seen, probably secondary to this congestion,

though it may have been the result of a very mild tuberculin reaction. One of these animals which had been sensitised by an inoculation with living tubercle bacilli was found at post mortem to have a cortical cerebellar tuberculoma. Yet in spite of a fatal dose of tuberculin intraperitoneally there was no generalised meningeal exudate. In view of these findings it seems likely that the so-called serous tuberculous meningitis (Lincoln, 1947) is due to the presence of a small number of tubercle bacilli in the subarachnoid space, rather than a sterile allergic reaction.

The nature of the substance which provokes the meningeal reaction is not finally settled. Other workers have mostly used old tuberculin, while we have been able to use the relatively pure Weybridge P.P.D., thus eliminating the glycerine broth as a possible non-specific irritant and much reducing the non-protein substances present. The preparation used contains 5 per cent polysaccharides (Green, 1946), and it is possible, though unlikely, that this is the active substance (McCarter and Watson, 1942). The nature of the antigen is, however, immaterial to the argument that the meninges become tuberculin-sensitive; the meningeal reaction is as specific as the Mantoux test and has the same right to be accepted as allergic in origin. We are convinced that in tuberculous guinea-pigs the meninges do become so sensitised, and in so far as it is also true of humans the reaction must play a part in the disease of tuberculous meningitis.

SUMMARY

The tuberculin reaction of the leptomeninges of guinea-pigs has been investigated. Purified Protein Derivative (P.P.D., Weybridge) was injected into the cisterna magna of tuberculin-sensitive animals and the meninges examined histologically at varying intervals up to 48 hours afterwards. A cellular exudate was present in the meninges of tuberculin-sensitive animals. No exudate was present in non-sensitised animals, nor after an injection of saline. We therefore

EXPLANATION OF PLATES.

Sections of brain and leptomeninges 24 hours after intracisternal P.P.D., showing amount of exudate.

FIG. 1.—Basal cistern and choroidal fissure, sensitised guinea-pig (No. 4, H. & E. × 49).

FIG. 2.—Basal cistern and choroidal fissure, non-sensitised guinea-pig. Note lack of exudate (No. 16, H. & E., × 48).

FIG. 3.—Pons and basilar artery, sensitised guinea-pig. Note the subarachnoid exudate and cuffing of the penetrating vessels. (No. 4, H. & E., × 48.)

FIG. 4.—Pons and basilar artery, non-sensitised guinea-pig. No exudate. (No. 15, H. & E., × 48.)

FIG. 5.—Transverse section through brain tesm and cerebellum of a sensitised guinea-pig showing the extent of the exudate around the brain stem and over the cerebellum. A higher power view of the exudate in this animal is shown in Fig. 3. (No. 4, H. & E., × 6·8.)

FIG. 6.—Mixed mononuclear and polymorphonuclear exudate in the subarachnoid space, seen 24 hr. after intracisternal injection of P.P.D. (No. 3, H. & E., × 780.)

FIG. 7.—Predominantly mononuclear exudate in the subarachnoid space 48 hr. after intracisternal injection of P.P.D. (No. 49, H. & E., × 780.) Macrophages engulfing polymorphs.

FIG. 8.—Mononuclear exudate in the subarachnoid space 8·5 hr. after an intracisternal injection of P.P.D. (No. 46, H. & E., × 780.)

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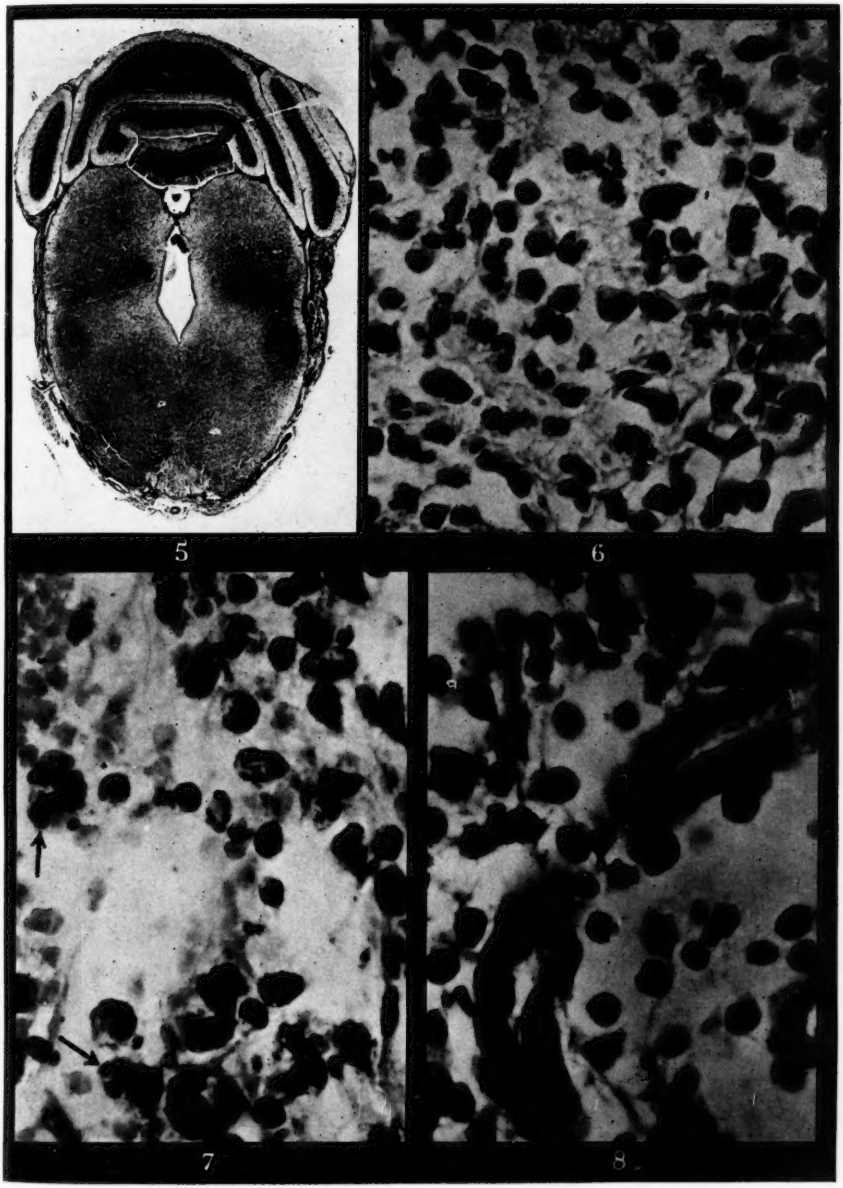

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concluded that the leptomeninges of guinea-pigs are capable of giving a tuberculin reaction.

The reaction in the meninges is predominantly mononuclear in character. It is not produced by intraperitoneal injections of tuberculin.

Tubercle bacilli appear to cause a similar reaction in 24 hours in both sensitised and non-sensitised guinea-pigs.

The relationship of this reaction in guinea-pigs to tuberculous meningitis in man is briefly considered.

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THE DEPOSITION OF ^{90}Sr IN RABBIT BONES FOLLOWING INTRAVENOUS INJECTION.*

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OBSERVATIONS on the deposition of ^{89}Sr in the skeleton of rabbits at different times up to 21 days after a single intravenous injection have already been reported (Kidman, Rayner, Tutt and Vaughan, 1952 ; Tutt, Kidman, Rayner and Vaughan, 1952). These results suggested that for a proper estimation of radiation hazards from radioactive strontium a long-term study should be made. Because of its relatively short half life ^{89}Sr is unsuitable for this purpose, since doses needed to give an autoradiograph might cause radiation damage. A long-term study was therefore undertaken using ^{90}Sr, an isotope with a half life of 20 years.

EXPERIMENTAL.

Rabbits in two age groups were studied, (i) 1 day old at the time of injection, (ii) 5–7 weeks old at the time of injection. Animals came from the same stock as previously used and were fed a diet of oats, hay and cabbage (Kidman, Tutt and Vaughan, 1950).

(i) *Rabbits 1 day old.*

A single intraperitoneal injection of $^{90}SrCl_2$ in carrier-free solution was given to 29 rabbits in doses varying from 20–40 μc. per kg. body wt. They were killed 24 hr., 9 days, 25 days, 6 weeks, 7 weeks, 8 weeks and 6 months later. Total retention in the skeleton was estimated and autoradiographs prepared from sections of the tibia-fibula.

(ii) *Rabbits aged 5–7 weeks.*

A single intravenous injection of $^{90}SrCl_2$ was given to 28 rabbits. They were killed in groups of two to six, 10 minutes, 24 hours, 9 days, 8 weeks, 16 weeks, 6 months, 12 months and 18 months later. The dose varied from 25–1000 μc. per kg. body wt. The majority received the smaller dose. Gain in weight and in length of long bones, the percentage of the dose retained, and the blood picture were unaffected by variations in the dose within this range. (Two additional rabbits given the biggest dose died unexpectedly. No cause was found at post-mortem. The results are not included.) Immediately after death the bones were removed as previously described. (Kidman *et al.*, 1952 ; Tutt *et al.*, 1952.) Sections were made from the right femur and right tibia for the preparation of autoradiographs. The left tibia and sometimes the right humerus were used for determination of ^{90}Sr retention in different parts of the bone. Retention in the scapula, pelvis, left femur and radius-ulna was determined separately and that of the rest of the skeleton together.

Estimations of ^{90}Sr.

Estimation of ^{90}Sr in the bones was carried out as described for ^{89}Sr except that all samples were left for 20 days to allow restoration of equilibrium (Tutt *et al.*, 1952). In the

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older animals it was only possible to divide the bone into head and shaft owing to closure of the epiphysis. The amount of isotope found is expressed throughout as a percentage of the injected dose rather than as percentage per gramme of bone, since in younger animals the growth dilution factor is considerable. It must, however, be remembered in looking at the tables that though the percentage retained in any two bones may be the same, the concentration of isotope may be different. The percentage retained is in part at least in certain age groups related to the initial uptake, but the growth dilution factor varies from bone to bone. In the tables therefore bone weight as well as percentage of injected dose retained is recorded.

Autoradiographs were prepared from sections of bone as previously described (Kidman *et al.*, 1952), all sections being embedded in methyl methacrylate monomer. Owing to variations in dosage and in the character of the X-ray stripping film available, exposure time could not be kept constant. Exposure was regulated to give a satisfactory macroscopic picture without suppressing or over-emphasizing any feature. Degrees of blackening in different autoradiographs cannot be compared, though differences in different parts of one autoradiograph are significant. Sections of normal bones containing no radioactive isotope were exposed as controls and were always negative.

RESULTS.

Total Retention in the Skeleton.

Maximum retention was observed in both age groups 24 hr. after injection (Table I), when the younger rabbits retained an average of $75{\cdot}1\pm1{\cdot}6$ per cent

TABLE I.—*Mean Values for ^{90}Sr (expressed as Percentages of the Injected Dose) Retained in the Skeleton of Rabbits Killed at Varying Time Intervals after Injection.*

Time after injection.	Age of rabbits.	
	1 day.	5–7 weeks.
10 minutes	—	$43{\cdot}1\pm0{\cdot}8$*
24 hours	$75{\cdot}1\pm1{\cdot}6$	$66{\cdot}6\pm3{\cdot}8$
9 days	$57{\cdot}1\pm6{\cdot}6$	$46{\cdot}1\pm4{\cdot}2$
25 ,,	43·0 —	—
6 weeks	$37{\cdot}2\pm4{\cdot}5$	—
7 ,,	34·4 —	—
8 ,,	$30{\cdot}2\pm1{\cdot}6$	$21{\cdot}9\pm3{\cdot}1$
16 ,,	—	$22{\cdot}0\pm0{\cdot}5$
24 ,,	$15{\cdot}1\pm2{\cdot}3$	$18{\cdot}8\pm1{\cdot}0$
52 ,,	—	$8{\cdot}9\pm0{\cdot}6$
18 months	—	$11{\cdot}0\pm0{\cdot}7$

* S.E. $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$ x = individual observation. $\bar{x}$ = mean of observations. n = number of observations in group.

and the older rabbits $66{\cdot}6\pm3{\cdot}8$ per cent of the injected dose. Loss of strontium from the skeleton then occurred. This was possibly rather more rapid in the younger age group. Six months after injection they retained only $15{\cdot}1\pm2{\cdot}3$ per cent of the injected dose as compared with $18{\cdot}8\pm1{\cdot}0$ per cent in the older animals. Eighteen months after injection the latter group still retained $11{\cdot}0\pm0{\cdot}7$ per cent of the initial dose.

Retention in Different Bones in Rabbits aged 5–7 Weeks.

There was no significant difference in the concentration of strontium in the long and flat bones 10 min., 24 hr. and 9 days after injection (Table II). After this both the absolute quantity, *i.e.*, total retention, and the concentration per g.

TABLE II.—*Weights of Bones and Percentages of Injected ^{90}Sr Retained at Different Times after Injection in 5–7-Week-old Rabbits.*

Time after injection.	Femur.		Radius-ulna.		Scapula.		Pelvic girdle.		Rest of bone.	
	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes	6·39±0·25	4·42±0·19	2·22±0·28	1·68±0·03	1·79±0·11	1·20±0·07	3·66±0·11	2·74±0·38	53±1·8	26·4±0·7
24 hours	6·62±0·19	7·46±0·33	2·52±0·09	3·24±0·40	1·84±0·01	1·79±0·04	4·29±0·05	4·41±0·24	56±1·3	38·9±2·7
9 days	5·38±0·16	4·82±0·43	1·92±0·11	1·68±0·24	1·66±0·14	1·21±0·15	3·50±0·15	3·00±0·33	46±0·7	28·2±2·5
8 weeks	10·36±0·67	2·92±0·29	3·66±0·09	0·98±0·11	3·45±0·17	0·57±0·07	7·56±0·30	1·39±0·19	93±1·4	12·1±2·1
16 ,,	12·3 ±0·40	2·85±0·09	5·30±0·30	1·07±0·05	4·20±0·20	0·52± —	8·80±0·20	1·33±0·05	111±1·9	12·3±0·7
24 ,,	10·5 —	2·11 —	5·20±0·25	0·84±0·02	4·80±0·45	0·49±0·10	9·10±1·01	1·06±0·12	114±6·8	10·3±1·0
12 months	13·0 ±0·90	1·51±0·10	5·20±0·20	0·52±0·04	5·50±0·90	0·19±0·02	11·30±1·70	0·52±0·03	136±5·2	4·1±0·4
18 ,,	16·0 ±0·58	1·65±0·11	6·30±0·33	0·57±0·04	7·17±0·17	0·23±0·03	13·30±0·67	0·66±0·04	143±2·3	5·8±0·4

TABLE III.—*Weights of Portions of Tibia-fibulas and Percentages of ^{90}Sr Retained at Different Times after Injection in 5–7-Week-old Rabbits.*

Time after injection.	Epiphysis.		Epiphyseal plate.		Metaphysis.		Diaphysis.	
	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes	0·184±0·013	0·30±0·02	0·223±0·012	0·85±0·02	0·281±0·019	0·45±0·04	0·492±0·014	0·33±0·003
24 hours	0·222±0·018	0·39±0·04	0·277±0·023	1·22±0·07	0·353±0·011	0·92±0·08	0·518±0·021	0·68±0·03
9 days	0·213±0·014	0·33±0·04	0·198±0·011	0·29±0·03	0·253±0·010	0·55±0·07	0·373±0·007	0·71±0·06
8 weeks	0·351±0·027	0·09±0·01	0·382±0·032	0·08±0·03	0·447±0·022	0·07±0·01	1·188±0·045	0·80±0·08

of bone, decreased more rapidly in the flat bones than in the long bones. If the amount of ^{90}Sr in the skeleton 24 hr. after injection, when maximum retention was found, is compared with the percentage of this maximum retained 18 months after injection, 22 per cent of the ^{90}Sr remained in the femur and only 13 per cent in the scapula. In the same time the weight of the femur had increased by 240 per cent while that of the scapula had increased by 390 per cent.

Retention in Different Parts of the Same Long Bone in Rabbits aged 5–7 Weeks.

Distribution in different parts of the humerus and tibia-fibula followed the same pattern. Figures for the latter only are given. The greatest quantity was present in the epiphyseal plate 10 min. after injection, and the least in the epiphysis and diaphysis; the plate still retained most and the epiphysis least 24 hr. after injection; between 24 hr. and 8 weeks after injection the epiphysis, plate and metaphysis lost a considerable proportion of their ^{90}Sr, while a slight increase occurred in the diaphysis. The loss from the plate was greatest between the 24-hr. and 9-day interval, that from the metaphysis between 9 days and 8 weeks (Table III).

Examination of the quantities in " ends " and diaphysis showed that between 8 weeks and 18 months after the injection there was a gradual loss of ^{90}Sr by the " ends," while the diaphysis accumulated more ^{90}Sr up to 16 weeks after injection, and thereafter lost very little (Table IV).

TABLE IV.—*Weights of " Ends " and Diaphysis of Tibia-fibulas and Percentages of ^{90}Sr Retained at Different Times after Injection in 5–7-Week-old Rabbits.*

Time after injection.	" Ends."		Diaphysis.	
	Weight (g.).	Per cent.	Weight (g.).	Per cent.
10 minutes	0·688±0·03	1·59±0·04	0·492±0·01	0·33±0·003
24 hours	0·851±0·02	2·53±0·13	0·518±0·02	0·68±0·03
9 days	0·680±0·03	1·20±0·17	0·373±0·01	0·71±0·06
8 weeks	1·179±0·05	0·24±0·02	1·188±0·05	0·80±0·08
16 „	1·124±0·13	0·17±0·02	1·782±0·05	0·89±0·07
24 „	1·153±0·08	0·19±0·03	1·996±0·11	0·89±0·07
12 months	1·160±0·09	0·08±0·002	2·139±0·13	0·49±0·03
18 „	1·586±0·06	0·15±0·02	2·281±0·02	0·75±0·04

Autoradiographs.

Rabbits aged 1 day.—Autoradiographs prepared from the long bones of rabbits killed 24 hr. after injection show a heavy reaction throughout mineralized bone (Fig. 1), with localized concentration beneath the epiphyseal plate and what appears to be a single localized line right down the shaft. An autoradiograph prepared from a section of a tibia 2 weeks after injection shows considerable reaction throughout mineralized bone, but no concentration in the region of the plate. There is a localized diagonal line beneath the metaphysis, and external to it a bow-shaped line which merges with the diagonal line at the lower metaphyseal region (Fig. 2). Two short diagonal lines below the metaphysis are visible, but no definite external curved line is seen in autoradiographs prepared from bones of animals killed 25 days after injection. The same is true of autoradiographs prepared from bones of animals killed 8 weeks after injection (Fig. 3). There is a considerable reaction in the trabeculae of the epiphysis and directly

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beneath the epiphyseal plate 24 hr. after injection. This is less clear 2 weeks after injection, and there is a diffuse reaction only throughout the trabeculae of the metaphysis comparable with that in the diaphysis above and external and internal to the diagonal lines. It is noticeable that in autoradiographs of bone removed 8 weeks after injection there is a considerable diffuse reaction extending above and below the diagonal lines in the corticalis and up into epiphysis and metaphyseal trabeculae. When these lines were laid down the bone measured 18·5 mm., whilst 8 weeks later it measured 60·6 mm. Six months after injection no autoradiograph was obtained.

Rabbits aged 5–7 weeks.—The distribution of ^{90}Sr in the long bones of rabbits aged 5–7 weeks as shown in autoradiographs did not differ from that of ^{89}Sr. There was a diffuse reaction throughout mineralized bone with heavy localized areas of concentration beneath the epiphyseal plate, in the region of the endosteum, in the metaphysis and in the region of the periosteum in the centre of the shaft 10 min. after injection (Fig. 4 and 5). The localized concentration beneath the plate was lost 9 days later, and there was some displacement of the endosteal and periosteal lines (Fig. 6). Autoradiographs showed a slight reaction in the spongiosa of the epiphysis and a trace only in the metaphysis 8 weeks after injection, and both persisted up to 18 months (Fig. 7 and 8). In the length of the diaphysis the bands of strong localized reaction were still found 8 weeks after injection. Two were short diagonal lines with their metaphyseal ends nearer the outer surface of the bone ; the third was curved and overlapped externally the two diagonal lines in the centre of the diaphysis (Fig. 9). The distance of the two diagonal lines from the epiphysis was greater at the more rapidly growing end of the bone. In successive autoradiographs taken at different times up to 18 months (Fig. 10, 11, 12) after injection, the diagonal and curved lines persisted but became somewhat closer together, and if the autoradiograph was left on the bone section the outer curved line was observed to have moved towards the centre of the corticalis. Between the lines there was a diffuse reaction, and extending from the diagonal lines towards both epiphyses a fainter diffuse reaction became apparent when more sensitive stripping film became available.

DISCUSSION.

The total percentage of ^{90}Sr retained in 5–7-week-old rabbits 10 min., 24 hr. and 9 days after injection agrees satisfactorily with the corresponding figures for retention of ^{89}Sr (Tutt *et al.*, 1952). There were, however, in some cases considerable variations in retention between different rabbits in the same group, although all rabbits were of the same stock and age, and had been fed alike. These variations do not appear to be related to differences in size, weight, rate of growth or to the amount of ^{90}Sr injected. This variability occurred particularly in rabbits killed 8 weeks after injection which were injected in two groups at different times. Two rabbits in one group retained 29·4 per cent of the injected dose, three rabbits in the second group $16{\cdot}9 \pm 0{\cdot}59$ per cent. There was no apparent reason to discard either group. These results have therefore been averaged. The mean retention in animals killed one year after injection appears unexpectedly low, but again no explanation can be given. It would clearly be desirable to repeat these observations using a larger number of animals in each group.

The more rapid loss of ^{90}Sr from the flat bones as compared with the long bones seems to be associated with a greater activity of metabolism in the former as exemplified by more rapid growth and weight gain.

^{90}Sr has a short-lived radioactive daughter product ^{90}Y. Since the time between killing the animal and seeing the finished autoradiograph could not, for technical reasons, be less than 5 weeks, there was no possibility of any feature in the autoradiograph being due to ^{90}Y present in the injection fluid or to that which might have migrated during life from the decay of ^{90}Sr. The picture seen resembled that found after an injection of ^{89}Sr, and was not like that seen after an injection of radioactive yttrium (Rayner, Tutt and Vaughan, 1953). It must be assumed that the present autoradiographs were dependent on ^{90}Sr with ^{90}Y remaining *in situ*.

Concentration of ^{90}Sr as of ^{89}Sr (Kidman *et al.*, 1952) occurs in sites of active bone growth.

In extremely young animals when growth is rapid, these areas of concentration are lost within a period of weeks and a diffuse redistribution of the radioactive isotope throughout the freshly formed bone is found. In animals aged 5–6 weeks at the time of injection the concentration in the metaphysis is lost within 2 weeks owing to rapid growth in length. The concentration in endosteal and periosteal bone formed at the time of injection, though displaced as the bone grows in length, is not lost, since growth in diameter of the bone is not sufficient to lead to its resorption. In this age group considerable redistribution of the radioactive material from the metaphyseal trabeculae occurs.

Chemical analysis of the different parts if the tibia-fibula confirms that there is a rise in the radioactive strontium content of the diaphysis while the epiphysis, epiphyseal plate and metaphyseal plate are losing activity. The strontium content of the whole bone decreases during this period. There is a redistribution of ^{90}Sr due presumably to the normal processes of resorption and apposition resulting in liberation of radioactive strontium into the circulating blood, whence some is excreted and some incorporated again in newly formed bone. This occurs most obviously in the bones of animals 24-hr. old when injected, but also in the bones of older animals. Total retention in any bone at any one time consists in part of strontium originally deposited—" primary deposition "—and in part of that reincorporated —" secondary deposition."

It might be argued that the concentrated endosteal and periosteal lines in the youngest age group are redistributed by movement within the bone by some at present unrecognized mechanism, but such a method of redistribution within bone cannot account for the removal and reincorporation from the lower ends of the metaphyseal trabeculae which appears to occur in both age groups. The fact that the autoradiographs prepared from bones of animals killed six months after injection were negative is not surprising, since the amount of ^{90}Sr present in the whole skeleton at this time was only 0·4 μc. per kg. A bigger dose given to the 24-hr.-old animals might well have caused radiation damage.

The present observations emphasize the importance of combining autoradiographic and chemical studies of isotope deposition in bone. Though total retention in the skeleton or in any one bone may be the same in animals of different ages at a certain time after injection, yet the distribution within the bone and therefore the possible radiation damage may be different. For instance, total retention in rabbits aged 5–7 weeks and rabbits aged 24 hr. at the time of injection

were not widely different 8 weeks after injection, yet an autoradiograph of the femur in the older animals showed intense concentration of the isotope in the shaft, while in the younger animals there was no gross concentration, but an apparent diffusion of the isotope throughout the bone. The older age group is possibly more liable to tumour production than the younger, though the total burden of radioactive material is the same in both.

SUMMARY.

Observations on the retention and deposition of ^{90}Sr in the skeleton of rabbits aged 1 day and 5–7 weeks at the time of injection and killed at intervals up to 18 months later are recorded.

Immediately following injection in both age groups there is localized concentration in areas of active bone growth. In the younger age group such areas of

DESCRIPTION OF PLATES.

AUTORADIOGRAPHS PREPARED FROM LONGITUDINAL SECTIONS OF RABBIT BONES AFTER A SINGLE INTRAVENOUS INJECTION OF ^{90}Sr.

FIG. 1.—Tibia-fibula: injected 1 day old (20 μc./kg.), killed 24 hr. later.

Note (i) heavy diffuse reaction throughout mineralized bone; (ii) localized reaction in region of epiphyseal plate line throughout diaphysis. × 3.

FIG. 2.—Tibia-fibula: injected 1 day old (20 μc./kg.), killed 2 weeks later.

Note (i) diffuse reaction only in metaphysics; (ii) on left side upper diagonal line of localized nature below metaphysis; (iii) below this bowed line of localized reaction (in actual autoradiograph there is diffuse reaction on either side of bowed line and external to upper diagonal line); (iv) on right side apparent continuous line of localized reaction through length of shaft. × 3.

FIG. 3.—Tibia-fibula: injected 1 day old (40 μc./kg.), killed 8 weeks later.

Note (i) diffuse reaction throughout mineralized bone; (ii) two short diagonal lines of localized reaction near the centre of the shaft. × 3.

FIG. 4.—Cortical bone.

Note diffuse even reaction in centre bounded on either side by localized reaction (the latter, if autoradiographs had been left on bone, would overlie recent endosteal and periosteal bone).

FIG. 5.—Femur lower end: injected 5–7 weeks old (80 μc./kg.), killed 10 min. later.

Note (i) intense localized reaction beneath the epiphyseal plate, in the region of the endosteum in the metaphysis, in the region of the periosteum in centre of the diaphysis; (ii) diffuse reaction throughout mineralized bone. × 3.

FIG. 6.—Femur lower end; injected 5–7 weeks old (80 μc./kg.), killed 9 days later.

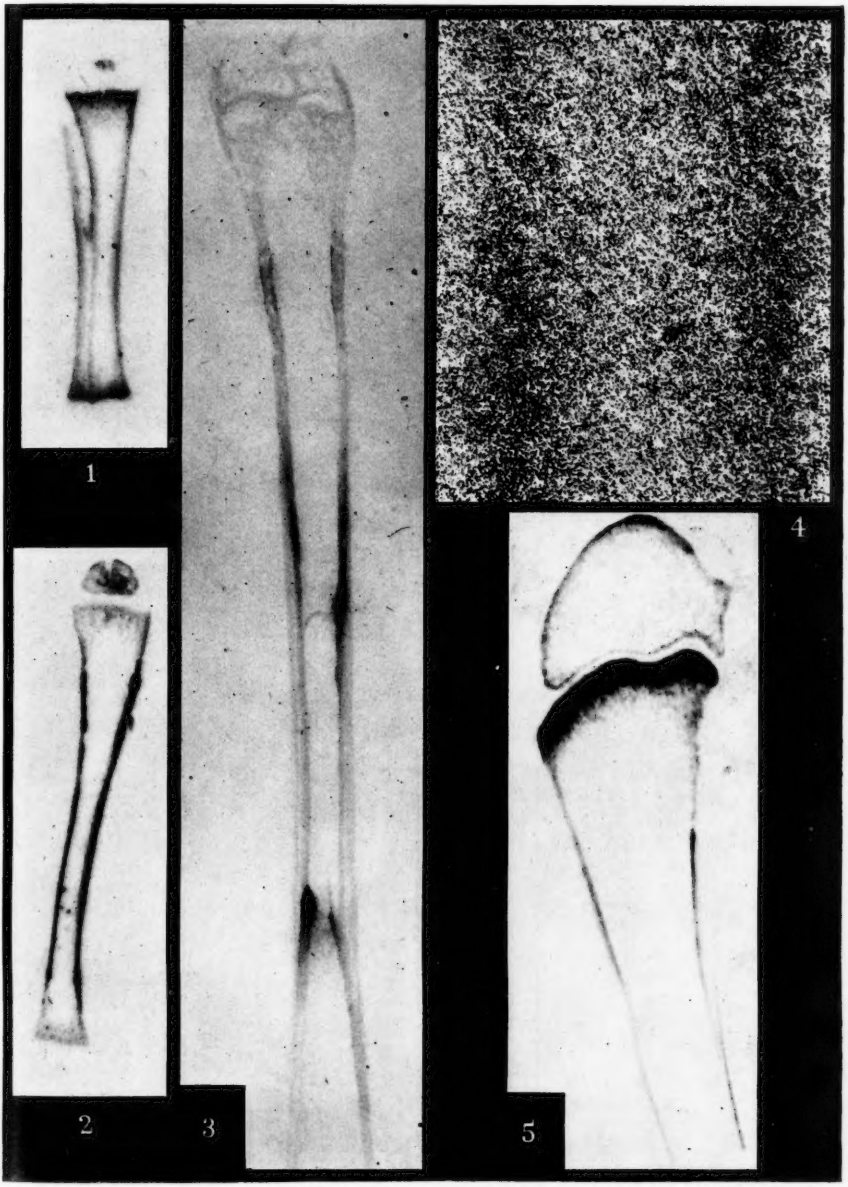
Note (i) diffuse reaction only in metaphyseal trabeculae at lower end; (ii) shift in localized lines in the diaphysis. × 3.

FIG. 7 and 8.—Femur lower end: injected 5–7 weeks old, killed 24 weeks (1000 μc./kg.) and 18 months (80 μc./kg.) later.

Note (i) persistence of lines of localized reaction in the diaphysis; (ii) diffuse reaction between the localized lines and in epiphysis; (iii) extremely faint diffuse reaction extending towards both epiphyses. × 3.

FIG. 9, 10, 11 and 12.—Tibia-fibula diaphysis: injected 5–7 weeks old, killed 10 minutes (80 μc./kg.) eight weeks (250 μc./kg.) 6 months (1000 μc./kg.) and 18 months (80 μc./kg.) later.

Note persistence of localized lines initially in the region of the endosteum and periosteum but gradual narrowing of the area of diffuse reaction between them.



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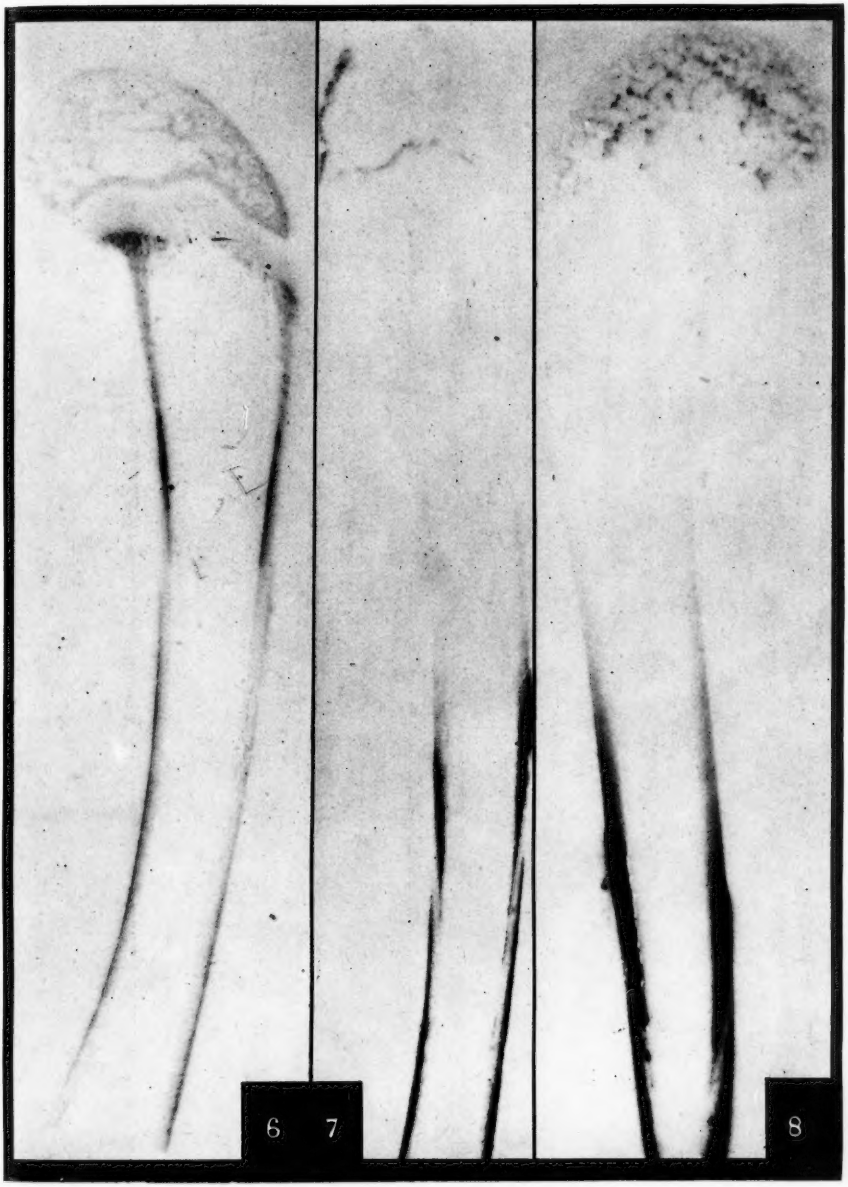

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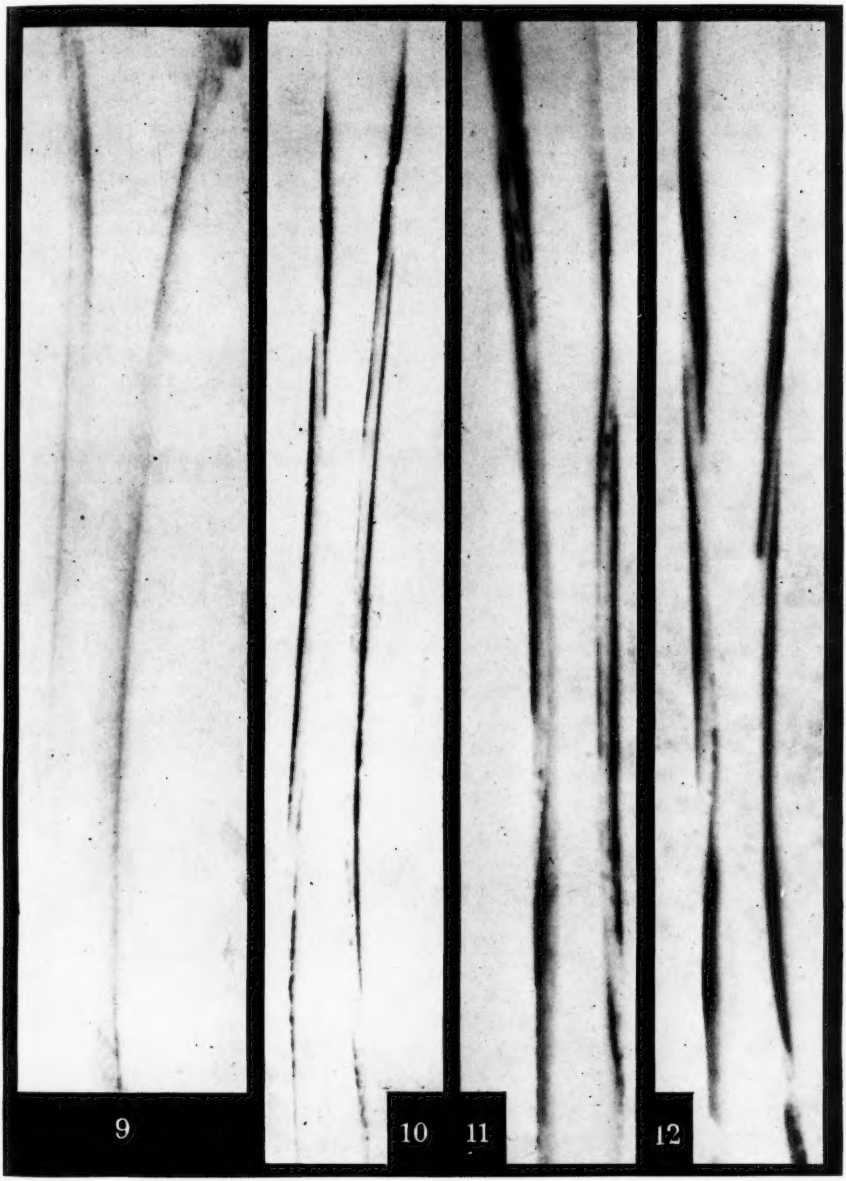

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concentration are almost entirely lost by the normal physiological processes of resorption and apposition. In the older group areas of concentration persist.

In the older animals the total amount of radioactive strontium retained in the skeleton at any one time is largely that incorporated at the time of injection (" primary deposition "), and to a lesser degree that taken up again from the blood stream after release from the bone by resorption (" secondary deposition ").

In younger animals the greater part of the strontium at any time after the first few weeks following injection is probably " secondary deposition " and is diffuse throughout the bone.

Total retention in bones from animals of different age groups may be the same, but the risk of tumour development may be different, since in one age group ^{90}Sr occurs largely in localized concentration, while in another deposition is macroscopically diffuse.

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UGANDA S AND YELLOW FEVER VIRUSES. A SLIGHT RELATIONSHIP SHOWN BY EXPERIMENTS IN RHESUS MONKEYS AND WHITE MICE.

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UGANDA S virus, isolated from *Aëdes* mosquitoes in Uganda, has been shown by immunity surveys to infect man in East Africa (Dick and Haddow, 1952). In 1950 during the course of a survey of immunity in sera collected from school children in Southern Nigeria, it was observed that there was a high percentage showing neutralisation against both yellow fever and Uganda S viruses. It was realised at the time that this correspondence of immunity could be due merely to the relatively high prevalence of the two infections. Nevertheless it was also observed that the sera of several of the laboratory rhesus monkeys which had been immunised against yellow fever, gave slight protection towards Uganda S in an intracerebral mouse protection test, whereas sera of unused "normal" monkeys gave no such protection. The phenomena observed might also have been produced if the stock Uganda S virus had been in some way contaminated with a strain of yellow fever. Procedures were therefore undertaken to eliminate any possible yellow fever virus from the preparation of Uganda S. During this process further evidence was produced which indicated a serological relationship between the two viruses.

An experiment was therefore planned to elucidate the situation. The number of rhesus monkeys available at the time was low, so that the experiment was restricted to a few animals only.

Two normal monkeys and two yellow fever-immune monkeys were inoculated with Uganda S virus. Their reactions, the presence of viraemia and the development of neutralising antibodies were observed. Subsequently all were challenged with a virulent strain of yellow fever virus and similar observations made. These experiments are described in Part I.

Another experiment was done in order to immunise white mice against one or other virus and challenge them with the heterologous strain. Part II deals with these observations.

PART I.

METHODS AND MATERIALS.

Virus of Uganda S.—This was kindly made available by Dr. K. C. Smithburn in the form of one ampoule of desiccated mouse brain. It was passed in mice by the intracerebral route, 24 animals receiving 1800 LD_{50}. The brains of seven mice which became sick 5 days after inoculation were harvested, and ground together to make a 5 per cent suspension in a pool of human serum received from the United States. After centrifugation at 2000 r.p.m. for 20 min., the supernatant virus suspension was desiccated.

All these procedures were carried out in a building separate from that in which work employing yellow fever virus was being undertaken. Nevertheless in order to eliminate

any yellow fever virus which might conceivably have been introduced during the preparation of the virus further passages were made, first through mice after incubation with yellow fever immune serum, and subsequently through a rhesus monkey which had been immunised against yellow fever.

For the first procedure serum from a yellow fever immune rhesus monkey, A627, was employed. The Neutralisation Index (see below) of the serum against yellow fever was 3200 when tested with normal human serum as a control.

One ampoule of desiccated Uganda S virus, one mouse passage removed from that received from Dr. Smithburn, was rehydrated in 2·5 ml. of saline to give a 10^{-2} mouse brain suspension. Further 10-fold dilutions were prepared in 10 per cent normal human serum in saline (10 per cent N.H.S.S.) and 0·3 ml. aliquots of virus dilutions were added to 0·3 ml. of the serum of A627 and to 0·3 ml. of normal human serum (N.H.S.) as a control titration. The mixtures were incubated at 37° for 2 hr., after which each was injected by the intracerebral route into a group of 6 adult white mice, each mouse receiving 0·03 ml. The mice were observed daily for 14 days. The mortality ratios are given in Table I. On the ninth day after inoculation a mouse in the group inoculated with the mixture of the immune serum A627 and the 10^{-4} dilution of virus became ill and was sacrificed. The brain was removed for further passage. Study of the titres of the mixtures shows that the yellow fever-immune serum neutralised 8 mouse LD_{50} of Uganda S virus compared with the N.H.S. control. The mouse, however, which was sacrificed for further passage was inoculated with material well (by 130 LD_{50}) within the neutralising power of the serum to neutralise yellow fever.

TABLE I.—*Mortality Ratio of Mice Inoculated with Uganda S Virus Mixed with Yellow Fever-Immune and Normal Sera.*

Serum.	Dilutions of Virus. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.	Titre.
A 627	6/6*	6/6	5/6†	1/6	0/6	0/6	—	1:31,700
N.H.S.	—	—	6/6	5/6	0/6	0/6	0/6	1:251,000

* The mortality-ratio is shown as a fraction, of which the numerator indicates the number of mice dying, and the denominator indicates the number of mice which were inoculated and surviving on the third day after inoculation.

† One sick mouse was sacrificed.

After one further mouse brain passage the strain was subjected to the second procedure of injection into a yellow fever-immune rhesus monkey. The monkey A626, adult male *M. mulatta*, had been inoculated subcutaneously with 3 million mouse i.c. LD_{50} of a viscerotropic strain of yellow fever 3 years previously and had survived, as sometimes occurs when a very high dosage is employed. Before inoculation with the Uganda S virus, serum of this animal gave Neutralisation Indices against neurotropic yellow fever and Uganda S of 450 and 7 respectively when normal rhesus serum was used as a control.

The monkey was inoculated subcutaneously with 1 ml. of a 10^{-4} mouse brain suspension containing 10,600 mouse LD_{50}. From the 2nd to the 7th day after inoculation the monkey was bled from the femoral vein and the serum injected intracerebrally into mice. Two mice which had been inoculated with serum taken on the fourth day after the inoculation of the monkey and which became sick after an incubation period of eight days were sacrificed for further passages. The brains of mice becoming sick on the second subsequent passage were ground in N.H.S. to make a 10 per cent suspension. This suspension was centrifuged at 2000 r.p.m. for 20 min. and the supernate, designated 10 per cent mouse brain, desiccated in ampoules containing 0·5 ml. each. This standard preparation was used in all subsequent experiments described below.

Virus of yellow fever.—For neutralisation tests a desiccated 10 per cent mouse brain prepared similarly to that of Uganda S was used, the virus being a neurotropic strain of yellow fever.

For the challenge of the monkeys with viscerotropic yellow fever the Asibi strain was used. This strain had been stored as the desiccated serum of a rhesus monkey which had been inoculated with Asibi virus and bled during the height of the illness.

Titration of virus.—The dosage of virus administered to the monkeys was determined by titration of the preparation in white Swiss mice bred in the Institute animal colony. Ten-

fold dilutions of the virus in 10 per cent N.H.S.S. were injected by the intracerebral route into groups of 6 mice. The mice were observed for 14 days and the mortality recorded. The 50 per cent end-point was calculated by the method of Reed and Muench (1938).

Neutralisation tests.—Ten-fold dilutions of virus suspension were made in 10 per cent N.H.S.S. and 0·3 ml. amounts were added to 0·3 ml. of serum to be tested and control serum. The control serum was either the pre-experimental serum of the monkey concerned or, where this was not available the serum of a normal rhesus monkey A622. (This serum when compared in a neutralisation test with the pre-experimental serum of rhesus A646 gave neutralisation indices of 0·45 and 0·72 for Uganda S and yellow fever viruses respectively). The serum-virus mixtures were incubated at 37° for 2 hr. and injected separately into two groups of six mice by the intracerebral route. The serum-virus mixtures and the inoculations were made in parallel so that the same dilution of virus in the sera was exposed to the same incubation period. The titres of the virus in the different sera were calculated, and the ratio between the titre of the virus in the control serum and that in the test serum is the Neutralisation Index (N.I.).

Experimental monkeys.—The following adult male *M. mulatta* monkeys were employed:

A646.—Not previously inoculated.
A647.— „ „ „
A653.—Jan., 1948, inoculated with 17D yellow fever virus.
„ —March, 1948 „ „ viscerotropic yellow fever virus.
A654.—Nov., 1947, „ „ neurotropic „ „ „
„ Feb., 1948, „ „ viscerotropic „ „ „

Tests for circulating virus.—Blood was collected from the posterior saphenous vein and the serum separated. The serum was injected intracerebrally into mice 45–60 min. after collection. Titrations were performed as described above.

Specificity tests, conducted on lines similar to a neutralisation index determination with the exception that a known specific immune serum was employed, confirmed that in every case the virus recovered was similar to that which had previously just been inoculated into the monkey concerned.

Experimental procedure.—The four monkeys were inoculated on April 25, 1951 (day 0) by the subcutaneous route with 0·5 ml. of a 10^{-5} mouse brain suspension in 10 per cent N.H.S.S. of the standard preparation of Uganda S described above containing 3320 mouse i.c. LD_{50}. Tests for circulating virus were made on the next nine days.

On May 19, 1951 (day 24) a booster inoculation of Uganda S was given, each monkey receiving by the subcutaneous route 0·5 ml. of a 10^{-3} mouse brain suspension containing 170,000 mouse i.c. LD_{50}. Tests for circulating virus were made 2, 4, 6, 8 and 10 days after inoculation.

On July 24, 1951 (day 90) each monkey was inoculated by the subcutaneous route with 0·25 ml. of a 10^{-3} dilution of serum containing 630 mouse i.c. LD_{50} of Asibi strain yellow fever virus. Tests were made for circulating virus and the monkeys observed for illness.

Specimens of sera were collected from the monkeys on days 0, 24, 90 and 120 and stored frozen until subjected to neutralisation tests.

RESULTS.

After the initial inoculation with Uganda S virus none of the monkeys showed any signs of sickness nor was the temperature raised above 104° F. at any time.

The amount of circulating virus was low, and there was no significant difference in the behaviour of the virus in normal and yellow fever immune monkeys (Table II).

After the booster inoculation of Uganda S no circulating virus was demonstrated in any of the monkeys.

After inoculation with the virulent viscerotropic Asibi strain of yellow fever the two immune monkeys showed, as was to be expected, no signs of illness, nor was viraemia demonstrated although rhesus A653 had a temperature of 104·2° F.

TABLE II.—*The Presence of Circulating Uganda S Virus in the Serum of Rhesus Monkeys Shown by the Mortality Ratios in Mice.*

	Days after inoculation.								
	1.	2.	3.	4.	5.	6.	7.	8.	9.
A646	2/11	0/12	2/12	2/12	0/12	0/12	0/12	0/12	0/12
A647	0/12	0/12	0/12	0/11	0/12	0/11	0/12	0/11	0/12
A653	0/12	0/12	0/12	0/12	*2/12	0/12	0/12	0/12	0/12
A654	0/12	0/12	1/12	1/12	1/12	0/11	0/12	0/12	0/12

* Not specific.

on the third and fourth days after inoculation. The two monkeys A646 and A647 immunised only by Uganda S virus both became sick. Their temperatures are given in Table III and the amount of the circulating virus in Table IV. One of these monkeys survived. The neutralisation indices of the sera taken throughout the course of the experiment are shown in Table V.

TABLE III.—*Rectal Temperature of Monkeys after Inoculation with Asibi Strain of Yellow Fever Virus.*

Monkey No.	Days after inoculation.								
	0.	1.	2.	3.	4.	5.	6.	7.	8.
A646	102·0	103·2	103·6	105·0	104·4	Died	—	—	—
A647	103·0	103·0	104·2	104·4	104·4	103·8	103·4	102·0	103·4
A653	103·2	103·4	103·6	104·2	104·2	103·8	103·8	103·8	103·8
A654	102·2	103·6	102·8	103·0	102·0	102·8	102·8	102·2	102·6

TABLE IV.—*Circulating Yellow Fever Virus.*

Monkey No.	Mouse LD_{50} in sera of Uganda S immune monkeys on specified day after inoculation.							
	2.	3.	4.	5.	6.	7.	8.	9.
A646	100	150,000	$>10^7$	$>10^7$	2×10^6	—	—	—
A647	1800	24,000	100,000	100	1	0	0	0

TABLE V.—*Neutralisation Indices against Uganda S (U.S.) and Yellow Fever (Y.F.) of Sera from Monkeys.*

Number of days after experiment commenced.	Monkey No.							
	A646.		A647.		A653.		A654.	
	U.S.	Y.F.	U.S.	Y.F.	U.S.	Y.F.	U.S.	Y.F.
0	0	0	0	0	470	3900	14	15,900
24	4	1·6	0	0	540	5000	1860	22,900
90	170	1·6	4·6	0	1660	7900	9550	14,800
120	37*	3·5*	1000	3500	1820	6900	5700	21,000

* Serum taken on day of death (96).

The results of the experiments in rhesus monkeys show that Uganda S virus is not pathogenic and has only weak antigenic properties in them. Immunity to Uganda S does not protect monkeys against yellow fever, although the survival of the one monkey when challenged with the highly virulent Asibi strain of yellow fever may indicate some modification of the course of the disease.

TABLE VI.—*Titration of Uganda S and Yellow Fever Viruses in Immunised and Control Mice.*

Challenge virus.	Mice immunised against.	Dilutions of virus. 10^{-2}. MR*	10^{-2}. AST†	10^{-3}. MR	10^{-3}. AST	10^{-4}. MR	10^{-4}. AST	10^{-5}. MR	10^{-5}. AST	10^{-6}. MR	10^{-6}. AST	10^{-7}. MR	10^{-7}. AST	10^{-8}. MR	10^{-8}. AST	10^{-9}. MR	10^{-9}. AST	Titre log. reciprocal of LD_{50}.
Uganda S.	Control	—	—	—	—	—	—	9/9	7·1	9/9	7·4	8/12	9·8	1/9	9·0	0/11	—	7·27
	Uganda S	—	—	0/9	—	0/9	—	0/11	—	0/7	—	0/11	—	0/7	—	—	—	<3·0
	Yellow fever	—	—	—	—	9/9	8·1	12/12	9·7	4/8	10·0	6/11	9·3	2/10	10·0	1/12	9·0	6·69
Yellow fever	Control	—	—	—	—	11/11	4·6	9/10	6·0	10/11	5·8	3/12	5·7	2/12	10·0	—	—	6·73
	Uganda S	—	—	10/10	5·4	6/6	6·7	7/11	6·6	3/9	5·3	3/9	7·7	0/8	—	—	—	5·70
	Yellow fever	3/12	5·3	5/5	6·2	5/12	13·3	7/12	6·6	4/10	8·0	0/12	—	—	—	—	—	4·78

* M.R. = Mortality ratio. The numerator represents the number of mice dying between the 4th and 14th day inclusive after inoculation. The denominator represents the number of mice inoculated and surviving on the third day.

† AST = Average survival time of mice before death. The number of mice inoculated with each dilution varied slightly owing to the fact that some had died as the result of the immunising inoculation, intercurrent infection or heat. Owing to this fact the titres have been calculated by the method of Thompson.

Immunity of the monkey to yellow fever does not appear to modify the infection with Uganda S. Nevertheless there is a definite though weak cross-relationship on serological examination.

Sera of monkeys immunised against Uganda S or yellow fever show some neutralisation of the heterologous virus, though this is much less than that of the homologous virus. The neutralisation of a Uganda S virus by a yellow fever immune serum is greater than the neutralisation of yellow fever virus by a Uganda S immune serum. This cannot, however, be attributed to the small amount of homologous antibody usually produced in a Uganda S-infected monkey, since, in a separate experiment, a rhesus monkey A652 which responded well to Uganda S and developed a neutralisation index of 15000 against this virus, had a neutralisation index of only 9 for yellow fever. In monkey A647 infection with yellow fever has greatly enhanced the neutralising power of the serum against Uganda S.

PART II.

Two groups of mice were immunised by the intraperitoneal injection of yellow fever or Uganda S virus. Titrations of the homologous and heterologous viruses were made in both groups. Control titrations were made in mice of the same stock and age which had been inoculated with a normal mouse brain tissue in a manner similar to those immunised by infective material.

METHODS AND MATERIALS.

Mice.—All mice were 35–40 days old at the start of the experiment. There were 150 in each of the 3 groups specified below.

Viruses.—Viruses of the same stock preparation as described in Part I were employed.

Immunising doses.

(*a*) *Uganda S.*—The mice were inoculated by the intraperitoneal route each with 0·03 ml. of a 1/1000 mouse brain preparation of Uganda S containing 4300 mouse LD_{50} on intracerebral titration.

(*b*) *Yellow fever.*—Intraperitoneal inoculation with 0·03 ml. of a 1/100 mouse brain preparation of neurotropic yellow fever virus containing 40,000 mouse LD_{50} on intracerebral titration.

(*c*) *Control group.*—Inoculated with a 1/100 brain preparation from healthy mice.

Challenge inoculation.—Fourteen days after the immunising dose titrations of Uganda S and yellow fever viruses were made in the immunised and control mice. The titrations were done as described in Part I. The inoculations of a dilution were made into all three groups of mice before the inoculation of the next dilution.

Owing to the fact that the number of mice inoculated for the varying dilutions was not constant the titres have been calculated by Thompson's method (Thompson, 1947).

RESULTS.

The results of the titrations are given in Table VI. If the ratios of the titres in the immunised and control groups are calculated, an immunisation index, analogous to a neutralisation index is obtained. These indices are presented in Table VII, from which it is seen that mice immunised with Uganda S virus

TABLE VII.—*Comparison of Log Immunisation Indices in Immunised Mice.*

Challenge virus.	Mice immunised against—	
	Uganda S	Yellow fever
Uganda S . . .	>4·27	0·58
Yellow fever . .	1·03	1·95

develop a marked resistance to the homologous virus and a slight immunity to yellow fever virus.

The results of the titrations in mice immunised against yellow fever are less satisfactory owing to the poor immunity developed by mice (Fox, 1943). Nevertheless a distinct immunity to the homologous virus is shown, and a study of the average survival time also indicates that some resistance has resulted to Uganda S virus as well.

DISCUSSION.

Smithburn (1952), using monkeys, did not find any cross protection in yellow fever and Uganda S immune sera. He was, however, ignoring cross protection where neutralisation indices of less than 100 were concerned. There is, moreover, a considerable degree of variation in the production of antibodies to Uganda S by individual monkeys, as has been shown by Dick and Haddow (1952) and by the experiments presented in this paper. Therefore the slight relationship could easily have been overlooked. Dick and Haddow state that further work is required on slight relationships.

The experiments described above indicate that there is a relationship between Uganda S and yellow fever, that the relationship is slight and that it is one-sided. The importance of slight relationships between viruses such as that between West Nile and Japanese B (Smithburn and Jacobs, 1942) and that between dengue and yellow fever has not been fully evaluated apart from the difficulties that may arise in diagnostic procedures. The marked increase in the neutralisation index for Uganda S in the serum of A647 after infection with yellow fever shows that further difficulties may arise in the interpretation of diagnostic procedures when tests are made on acute and convalesent phase sera.

Although the experiments reported in this paper apply only to the serology in monkeys, we have already confirmed that a similar picture can occur in man. A rise in the neutralisation index for Uganda S from 4– to 1500–fold with an average of over 300-fold has been found in the acute and convalescent phase sera of 6 patients, all Africans, who were observed during an epidemic of yellow fever and diagnosed by serology and isolation of virus.

Infection with Uganda S virus is relatively common in Southern Nigeria. The sera of 72 per cent of 32 children in South-Western Nigeria in intracerebral tests showed protection against 100 or more mouse LD_{50} and another 16 per cent against 50–100 mouse LD_{50}. Nevertheless, the clinical manifestations of the infection have not yet been recorded. Yellow fever also is common in Southern Nigeria, and of the same group of children 63 per cent were shown by mouse protection test to have had yellow fever, yet no yellow fever has been reported from that particular area for a period longer than the age of most of the school children. This situation could be attributed to poor medical facilities but this is not felt to be the true explanation. This may lie either in the fact that here yellow fever is a children's illness and that, as has been observed in this country, children do not suffer as much as adults, or that here the disease may be somewhat modified.

The survival of the one rhesus monkey A647 and the slight immunity to yellow fever of the Uganda S immunised mice suggests that previous infection with Uganda S may in humans have a modifying effect on the course of the disease of yellow fever. Further work is being undertaken on this aspect, and surveys have shown that the incidence of infection with Uganda S is lower in the savannah

country of Nigeria. It is moreover in the areas between the savannah and the forest that the serious and fatal epidemics of yellow fever usually occur.

The one-sided cross relationship between the two viruses has recently been shown by Kerr (1952) in complement-fixation tests. Using immune guinea-pig sera he demonstrated fixation of complement when Uganda S immune serum was mixed with yellow fever antigen but not when the alternative antiserum and antigen were mixed. This one-sided reaction is thus the reverse of that found in the neutralisation indices employing monkey sera. Mouse brain virus was used as the antigen by Kerr, and in our tests for neutralisation indices. Therefore it must be assumed that either the monkey and guinea-pig reacts to an antigen common to the two viruses but to different degrees according to whether the antigen is united with the Uganda S or yellow fever virus, or that there are two different antigens each attached in differing amounts to the two viruses.

SUMMARY.

Serological studies in monkeys show that there is a slight cross relationship between Uganda S and yellow fever. The relationship is one-sided, being more marked when yellow fever immune serum is employed. The behaviour of Uganda S virus in monkeys is confirmed.

White mice immunised against one of the two viruses show a slight resistance to the other virus.

Man and monkeys may show similar serological reactions to the two viruses.

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THE PATHOGENESIS OF INFECTIOUS MYXOMATOSIS: THE MECHANISM OF INFECTION AND THE IMMUNOLOGICAL RESPONSE IN THE EUROPEAN RABBIT (*ORYCTOLAGUS CUNICULUS*).*

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MYXOMATOSIS is a natural disease of *Sylvilagus brasiliensis* (syn. *minensis*) (Aragão, 1943), a native rabbit of Brazil, and probably of other rabbits of the same genus in other parts of America. In this species of rabbit it produces a localized tumour, not unlike the Shope fibroma in the cottontail or the European rabbit, and causes no appreciable mortality. In the European rabbit, on the other hand, it causes a generalized disease associated with an extremely high case-mortality rate, of the order of 99·5 per cent or more in previously unexposed populations (Fenner, Marshall and Woodroofe, 1953). Investigations on the mode of transmission of myxomatosis by mosquitoes showed that these insects, which were undoubtedly the principal vectors in the great Australian epizootics (Ratcliffe, Myers, Fennessy and Calaby, 1952), transmit the disease mechanically, and acquire virus only when they bite through epidermal cells containing a high concentration of virus (Fenner, Day and Woodroofe, 1952). Virus contained in the circulating blood appears to be unable to render the insects infectious. While the experiments upon which this conclusion was based were unequivocal, it did not appear easy to correlate the apparent frequency with which mosquitoes were infected with the comparative rarity in the field of rabbits with many large skin lesions, which would obviously provide a satisfactory source of virus for them. The experiments which form the basis of this report were undertaken to determine whether there was a more common source of virus in the skin than the single primary lesions and the rare multiple tumours, and to see if myxomatosis conformed in its mechanism of infection to other generalized virus diseases (Fenner, 1950). Concurrently, the immunological response of infected rabbits was studied, use being made of animals previously immunized with fibroma virus (Shope, 1932), and others infected with Hurst's neuromyxoma virus, a variant of reduced virulence (Hurst, 1937*b*).

MATERIALS AND METHODS.

Viruses.

Two strains of myxoma virus were used, the classical South American strain (Fenner, Day and Woodroofe, 1952) and Hurst's "neuromyxoma" (Hurst, 1937*b*). Dr. E. Weston Hurst supplied the latter strain.

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The OA strain of fibroma virus was provided by Dr. Richard E. Shope.

Stock virus was prepared from the subcutaneous tissue and testes of rabbits which had been given massive subcutaneous inoculations of the viruses. The gelatinous subcutaneous tissue was minced, ground thoroughly before and after freezing in a mortar, and taken up in normal rabbit serum. The suspension was distributed in a large number of ampoules, which were sealed and stored at −70°. This provided a source of virus of known high titre.

Rabbits.

Adult rabbits of various skin colours, weighing about 2·5 kg., were used. They had been bred in the animal house of the Walter and Eliza Hall Institute of Medical Research.

Mosquitoes.

Female *Aëdes aegypti* (*L*) mosquitoes were kindly provided by Dr. M. F. Day, of the Division of Entomology, Commonwealth Scientific and Industrial Research Organization. They were kept in a constant temperature room at about 27° and a relative humidity of 60–80 per cent.

Titration of the virus-containing suspensions.

Suspensions containing virus were titrated on the chorio-allantois of the developing chick embryo (Lush, 1937). Aliquots of the suspensions were stored in the dry ice cabinet, and if the chick embryo titrations were negative they were titrated by the intradermal inoculation of rabbits (Parker, 1940).

Chick embryos 11 or 12 days old were inoculated chorio-allantoically with 0·05 ml. of tenfold dilutions of the tissue suspensions, three eggs being used at each dilution. After three days' incubation at 35° the pocks on the membranes were counted, with the help of a binocular dissecting microscope if necessary. The titre is expressed as the average pock count per ml. of suspension.

Rabbits were inoculated intradermally in several sites with 0·1 ml. aliquots of the various dilutions used. Usually these were made in half log. or 2-fold (0·3 log.) steps. Readings were made on the third, fourth and fifth days, before the development of secondary lesions. The titre was calculated by the method of Reed and Muench (1938).

Neutralizing antibody.

Neutralizing antibody was titrated on the chorio-allantois of developing chick embryos. The technique of the test has been discussed in some detail in an earlier paper (Fenner, Marshall and Woodroofe, 1953). Undiluted serum, inactivated by heating for 30 min. at 56°, was mixed with the appropriate virus suspensions, and held at room temp. for 30 min. before inoculation of the chick embryos. All sera derived from any one experiment were titrated simultaneously.

Complement fixation.

The technique of the complement fixation test and the preparation of myxoma and control antigens was described earlier (Fenner, Marshall and Woodroofe, 1953). Serial 2-fold dilutions of each sample of serum were tested for complement-fixing activity in the presence of three 50 per cent haemolytic doses of complement and a standard dilution (usually 1/8 dilution of the 10 per cent suspension of infected chorio-allantoic membranes) of myxoma antigen. The indicator system of sensitized red cells was added after fixation had taken place overnight at 4°.

Soluble antigen in the serum was demonstrated by complement fixation tests. The sera were inactivated by heating at 62° for 15 min., and were diluted serially in 2-fold steps and titrated, in the presence of three 50 per cent haemolytic doses of complement, against a standard myxoma-immune serum, and normal rabbit serum, both at a dilution of 1/50. The indicator system was added after overnight fixation at 4°.

The anticomplementary activity of the serum samples was determined by the method described by Donnelley (1951). Undiluted sera were inactivated by heating at 62° for 15 min. To each was added an equal volume of excess complement (nine 50 per cent haemo-

lytic doses), and the system allowed to stand overnight at 4°. The " free " complement was then estimated by diluting out the mixture in 2-fold steps and adding to each dilution an equal volume of sensitized red cells. The end-point (50 per cent haemolysis) was read after incubation for 30 min. at 37°. Normal serum was used as a control and the anti-complementary activity of the sera was expressed as the percentage of complement fixed, taking the figure obtained with normal serum as zero.

Comparison of the sensitivity of rabbits and eggs to the virus.

Lush (1937) found that a suspension which produced an average count of 2·0 pocks on the chorio-allantoic membrane with an inoculum of 0·05 ml. of a 10^{-3} dilution killed 3/3 rabbits inoculated with 0·2 ml. of the 10^{-4} dilution and 1/3 of those inoculated with the 10^{-5} dilution ; i.e., the rabbit was somewhat more sensitive than the chick embryo. A more exact comparison was necessary for the present studies, and the following experiment was performed.

An ampoule of myxoma virus which had been stored at −70° and of which the approximate titre was known, was diluted in normal saline containing 10 per cent horse serum by 10-fold steps, using a clean 1·0 ml. pipette and 9 ml. blanks for each dilution. From 10^{-3}, 2-fold dilutions were made to $10^{-4.5}$, and twenty twelve-day-old chick embryos were inoculated chorio-allantoically with 0·05 ml. of the dilutions $10^{-3.6}$ $10^{-3.9}$ $10^{-4.2}$ $10^{-4.5}$. From 10^{-5} 2-fold dilutions were made to $10^{-7.1}$, and rabbits were inoculated with 0·1 ml. in each of twenty sites, one rabbit being inoculated with each suspension from dilutions $10^{-5.0}$ to $10^{-7.1}$. The results of the pock count on the chorio-allantoics are shown in Table I, and the results

TABLE I.—*Pock Counts on Chorio-allantoic Membranes Inoculated with Dilutions of a Standard Suspension of Myxoma Virus.*

Dilution.	Pock counts.	Mean.
$10^{-3.6}$	40, 49, 9, 36, 29, 25, 52, 34, 13, 8, 2, 2, 26, 34, 44, 31, 12	26·2
$10^{-3.9}$	1, 10, 21, 2, 4, 8, 9, 4, 29, 1, 21, 12, 2, 9, 7, 14, 6, 12	9·6
$10^{-4.2}$	1, 6, 0, 1, 12, 0, 1, 6, 11, 16, 14, 12, 5, 3, 1, 1, 4, 2, 5	5·3
$10^{-4.5}$	1, 2, 1, 1, 2, 3, 11, 7, 0, 1, 2, 3, 2, 2, 3, 1, 7, 6, 3, 0	2·9

12-day-old embryo eggs incubated for 3 days at 35° after inoculation.
Inoculum volume = 0·05 ml.

TABLE II.—*Results of Intradermal Inoculation of Rabbits with Dilutions of a Standard Suspension of Myxoma Virus.*

Dilution.	Positive.	Negative.	Percentage positive.
$10^{-5.0}$	20	0	100
$10^{-5.3}$	19	1	95
$10^{-5.6}$	19	1	95
$10^{-5.9}$	15	5	75
$10^{-6.2}$	14	6	70
$10^{-6.5}$	4	16	20
$10^{-6.8}$	1	19	5
$10^{-7.1}$	1	19	5

Inoculum = 0·1 ml. of dilutions used for egg titration (Table I).

of the rabbit inoculation in Table II. The latter were very similar to those reported by Parker (1940). If all the results in the egg experiment were pooled the figure was obtained of $10^{-6.25}$ pock-producing particles per ml. in the suspension, compared with a Reed and Muench titre of $10^{-7.27}$ $I.D._{50}$ per ml. in the rabbit. Since the mean number of infectious particles in one $I.D._{50}$ is 0·693, the rabbit titre is equivalent to $10^{-7.10}$ infective particles per ml. For the purposes of present experiments a comparative figure only was required, and in all the subsequent experiments the egg titre (infective particles per ml.) has been converted to $I.D._{50}$ per ml. (rabbit) by multiplying the former by 10.

RESULTS.

Distribution of Virus in Rabbits following Infection.

The plan of the first experiment followed rather closely that previously employed in studies on mousepox (Fenner, 1948*a*).

Twenty adult male rabbits were inoculated intradermally in a marked area in the left flank, in the centre of an area about 3 cm. in diameter from which the hair had been removed by plucking. Simultaneously the virulent myxoma virus used for the inoculations was titrated in eggs and in a rabbit. The dose of 0·1 ml. of the $10^{-4\cdot7}$ dilution was found to contain approximately 20 $I.D._{50}$ of virus.

At daily intervals one or two rabbits were bled from the heart, some of the blood being heparinized and the remainder used for serum collection. They were then exsanguinated by washing out the vascular system with 4 l. sterile normal saline through a cannula which was inserted through the left ventricle and into the aorta.

The virus content of the heparinized blood was determined with whole blood; washed cells and plasma were also tested separately. "Plasma" consisted of the final supernate after three cycles of centrifugation, each for 30 min. in an angle centrifuge; "washed cells" comprised the sediment of the first centrifugation washed once with normal saline, deposited by centrifugation for 30 min. and then made up to original volume of the blood in normal saline.

The skin was removed from the inoculated site, and from an equivalent area on the other side of the body. The hair was plucked from the latter site after the rabbit had been exsanguinated. The crural lymph-nodes beneath the skin at these two sites were removed, then the left testis, the spleen and the left lung. All organs and pieces of tissue were weighed and ground with sand in mortars which had been cooled at −20°. After the preliminary grinding the tissues were frozen at −20° before re-grinding. The final suspension was taken up in a volume of normal horse serum saline equivalent to the weight of the sample, or ten times this weight, depending on the size of the sample; a portion was ampouled and stored at −70°, and the rest was titrated in either or both eggs and the rabbit. The results are summarized in Table III. Certain data have been extracted from this table and used to construct Fig. 1. Each rabbit was carefully examined for abnormal physical signs, and from the 5th day onwards washings were made of the conjunctival fluid.

It is apparent that in myxomatosis, as in mousepox and other generalized viral and bacterial diseases, there is a stepwise invasion of the organs. After an initial period of multiplication in the site of inoculation, virus reached and probably multiplied in the draining lymph node, in which a small amount of virus could be demonstrated 48 hr. after inoculation. By the next day small amounts of virus could be detected in a number of organs and in the cells of the circulating blood. There was a steady rise in titre in all affected organs. Virus was first demonstrated in the testis and "normal" skin a day later than in the blood and central organs. The titre in the whole blood and washed cells was identical throughout, and virus was never demonstrated in the plasma. In the day or so before death the amount of detectable virus in certain organs (blood, spleen, etc.) fell somewhat.

Daily examination of the animals showed that changes were present in both the inoculated skin site (redness, thickening) and in the regional lymph node

TABLE III.—*Titre of Myxoma Virus in Various Organs and Tissues at Intervals after Inoculation, Expressed as the Log_{10} of the I.D.$_{50}$ per ml. of Fluid or g. of Tissue.*

	Days after inoculation.									
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Skin-inoculation site	2·6, –	2·8, 4·7	6·5, 5·9	7·2, 7·3	6·7, 7·3	7·6	8·3	8·3, 8·4	8·4, 8·0	7·7
"Normal" skin	—	—	0, 0	0, 2·4	2·1, 3·9	5·5	4·7	6·4, 7·2	7·1, 5·7	7·1
Lymph node: inoculated side	0, 0	2·6, 2·6	7·7, 5·8	7·3, 7·4	6·1, 6·8	7·9	6·4	6·9, 7·8	6·9, 6·7	6·8
Lymph node: "normal" side	—	0, 0	0, 2·3	3·1, 4·5	3·2, 3·1	4·5	4·8	4·5, 6·9	6·1, 5·1	6·6
Whole blood	0, 0	0, 0	2·9, 2·8	3·9, 3·8	1·6, 3·8	3·3	2·3	3·4, 5·5	2·0, 1·8	—
Washed blood cells	0, 0	1·3, 0	2·8, 2·7	3·3, 3·6	1·8, 3·6	3·3	1·9	3·0, 5·6	2·3, 1·8	—
Plasma	0, 0	0, 0	0, 0	0, 0	0, 0	0	0	0, 0	0, 0	—
Lung	0, 0	0, 0	3·2, 2·5	4·4, 4·3	4·3, 4·1	6·0	5·6	5·1, 7·4	4·3, 3·6	7·0
Spleen	0, 0	0, 0	3·1, 2·7	4·5, 4·2	4·2, 3·9	4·2	3·2	0, 5·5	0, 2·9	7·0
Testis	0, 0	0, 0	0, 0	0, 2·4	3·9, 4·5	5·1	7·4	8·1, 8·0	8·3, 7·4	7·4
Conjunctival washings	—	—	—	—	4·1, 4·2	4·7	5·5	5·1, 6·3	5·9, 6·6	7·9

– = not determined. 0 = < 10 I.D.$_{50}$ (lowest amount detectable).

TABLE IV.—*Development of Neutralizing and Complement-fixing Antibodies, and of Complement-fixing Antigen, in Rabbits Injected in 20 Sites with Small Doses of Myxoma Virus (20 I.D.$_{50}$) and Neuromyxoma Virus (10 I.D.$_{50}$).*

	Neutralizing antibody.[1]						Complement-fixing antibody.[2]						Complement-fixing antigen.[3]			Anticomplementary activity.[4]					
	Neuro-myxoma.		Myxoma.			Fibroma then myxoma.	Neuro-myxoma.		Myxoma.			Fibroma then myxoma.	Myxoma.			Neuro-myxoma.		Myxoma.			Fibroma then myxoma
Days after inoculation.	255.	257.	259.	260.	261.	262.	255.	257.	259.	260.	261.	262.	259.	260.	261.	255.	257.	259.	260.	261.	262.
1 day	0	0	0	0	0	67	0	0	0	0	0	40	0	0	0	0	0	0	0	0	0
4 days	0	0	0	0	0	65	0	0	0	0	0	40	0	0	0	0	0	0	0	0	0
5 „	40	42	0	5	45	76	0	0	0	0	0	40	0	0	0	0	0	0	13	33	38
6 „	35	38	32	0	0	96·2	0	0	0	0	0	640	0	0	0	7	0	0	88	78	33
7 „	0	0	0	0	0	98·2	0	0	0	0	0	640	2048	0	0	43	43	69	88	86	96
8 „	0	0	0	0	0	98·3	0	0	0	0	0	640	2048	2048	2048	50	76	75	94	94	98
9 „	29	29	29	50	0	98·7	0	0	0	0	0	1,280	2048	4096	4096	50	86	92	94	95	92
10 „	72	65	†	†	†	99·3	0	0	†	†	†	1,280	†	†	†	79	90	†	†	†	92
11 „	85	84	..	..	..	98	160	80	..	..	..	1,280	..	..	..	50	77	..	..	..	92
12 „	90	87	..	..	..	98	160	320	..	..	..	640	..	..	..	47	72	..	..	..	88
14 „	96·5	95·6	..	..	..	98·3	640	640	..	..	..	2,560	..	..	..	14	72	..	..	..	54
15 „	96·7	96	..	..	..	99	640	640	..	..	..	2,560	..	..	..	14	72	..	..	..	50
21 „	97·2	97	..	..	..	98·7	2560	2560	..	..	..	10,240	..	..	..	7	72	..	..	..	34
28 „	98·7	96·7	..	..	..	98·3	5120	5120	..	..	..	10,240	..	..	..	0	50	..	..	..	36

[1] Neutralizing antibody: percentage reduction in pock count on chorio-allantoic membrane.
[2] Complement-fixing antibody: reciprocal of titre.
[3] Complement-fixing antigen: reciprocal of titre. Neuromyxoma and fibroma-myxoma rabbits: no complement-fixing antigen at any stage.
[4] Anticomplementary activity = $100 - \frac{100 \times \text{titre in presence of anticomplementary substance (serum sample)}}{\text{complement titre (normal serum)}}$.

† = Death.

(enlargement, engorgement) on the 3rd day after inoculation. Slight thickening of the eyelids was detectable on the 5th day, and virus could then be isolated from washings of conjunctival fluid. On the 6th day there were visible on the skin opposite the inoculation site a number of macules about 3 mm. diameter, and examination of the rest of the carcass showed that these occurred generally.

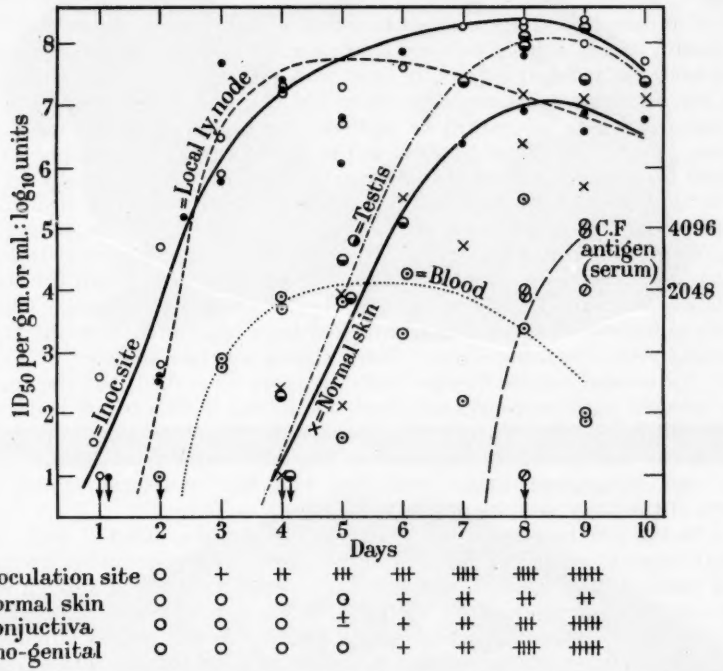

$1D_{50}$ per gm. or ml.: $\log_{10}$ units
Days
○=Inoc. site
●=Local ly. node
×=Normal skin
⊙=Testis
⊙=Blood
C.F antigen (serum)
4096
2048
Inoculation site o + ++ +++ +++ +++ ++++ ++++
Normal skin o o o o + ++ ++ ++
Conjuctiva o o o ± + ++ ++ ++++
Ano-genital o o o o + ++ +++ ++++

FIG. 1.—Concentrations of myxoma virus and of soluble complement-fixing antigen (ordinates on right = reciprocal of titre) in various tissues and organs of rabbits at intervals after the intradermal inoculation of 20 $I.D._{50}$ of virus, correlated with the development of abnormal physical signs.

± to +++++ = arbitrary grading of severity of physical signs.
"Normal skin" = skin on a portion of the body distant from the inoculation site.
Arrowed points = < 10 $I.D._{50}$/gm. (lowest amount detectable).

They became larger and more thickened with the passage of time. There is no doubt that they constitute a true secondary rash closely analogous to that characteristic of the acute exanthemata. Fig. 2 illustrates this rash in a rabbit 8 days after inoculation with 20 $I.D._{50}$ of virus. Areas of engorgement were noticed in the body of the testis from the 6th day onwards. The severity of all these signs increased until the time of death.

The Immunological Response in Infected Rabbits.

The serum collected from the 20 animals used for the experiment was tested for complement-fixing and neutralizing antibodies, complement-fixing antigen,

* Anticomplementary activity = 100 − 100 × titre in presence of anticomplementary substance (serum sample) / complement titre (normal serum).
† = Death.

and anticomplementary activity. All sera were tested together, so that results would be comparable. It appeared that the animals just began to produce antibodies a day or so before they died. Although no free complement-fixing antibody appeared, the occurrence of anticomplementary activity in the serum from the 7th day onwards was evidence of the presence of antigen-antibody union *in vivo*.

The immunological response was followed with greater ease in the next experiment, in which both myxoma and neuromyxoma viruses were used. This experiment was planned differently from that just described, the object being to trace the immunological response more clearly, and to compare the clinical symptoms and rate of growth of neuromyxoma virus with the fully virulent myxoma virus. The latter features will be discussed in a later paper.

With each strain of virus 3 rabbits were inoculated intradermally on marked sites in the carefully plucked skin of each flank, with a total of 10 inoculations each of 0·1 ml., the inoculum containing about 20 $I.D._{50}$ of myxoma virus and 10 $I.D._{50}$ of the neuromyxoma virus. The titres of the inocula were determined by making 10 inoculations of each of 4 appropriately selected 2-fold dilutions, and calculating the titre by the method of Reed and Muench (1938). One of the animals inoculated with myxoma virus had been inoculated with the OA strain of fibroma virus 8 months earlier. Each animal was bled daily.

All the normal rabbits infected with myxoma virus died, but the survival of those infected with neuromyxoma, and the animal (R262) which had been previously inoculated with fibroma virus, allowed a more satisfactory study to be made of the immunological response to infection. The development of neutralizing and complement-fixing antibodies and the complement-fixing antigen content of the daily samples of serum are shown in Table IV.

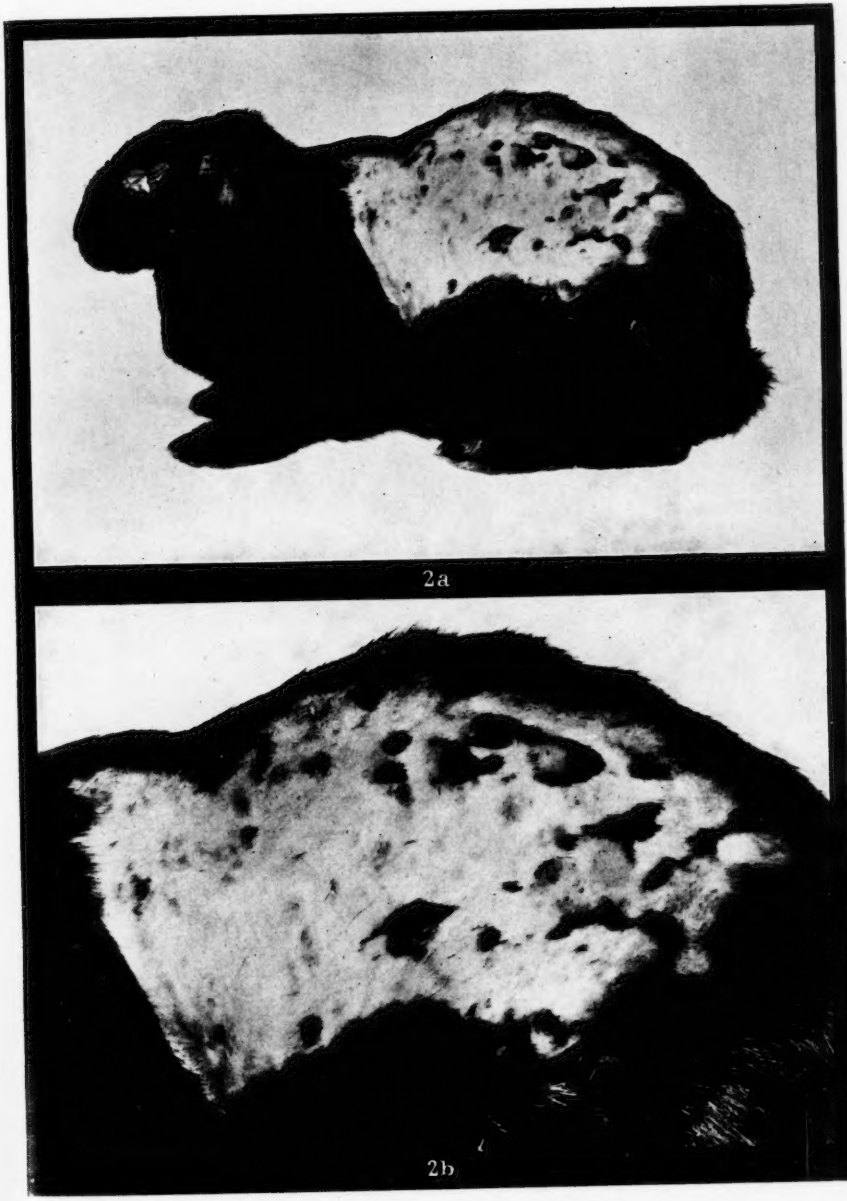
As in the previous experiment, the normal rabbits infected with the fully virulent myxoma virus (R259, R260, R261) produced no detectable free antibody before death, but on the 7th and 8th days complement-fixing antigen appeared in the serum in large amounts. From the 6th and 7th days there was a considerable degree of anticomplementary activity in the serum, which can be regarded as an index of the presence in the serum of an antigen-antibody complex (Donnelley, 1951).

In the rabbits infected with neuromyxoma the development of antibody could be clearly followed. Complement-fixing antigen could not be detected at any stage, but anticomplementary activity appeared on the 7th day and persisted until the 28th day. A significant rise in neutralizing antibody occurred

EXPLANATION OF PLATE.

FIG. 2A.—Photograph of a rabbit which had been inoculated intradermally in the right flank 8 days earlier with 20 $I.D._{50}$ of myxoma virus. The area on the left flank was plucked just before the photograph was taken, and shows, besides some patches of black hair, numerous secondary papules which were red in colour and varied from 2–10 mm. in diameter. These are seen more clearly in the enlargement of the plucked area of skin (Fig. 2B). The generalized swelling of the face, closure of the eyes, with a purulent conjunctival discharge, and thickening and drooping of the ears are characteristic of advanced myxomatosis. This rabbit was used immediately afterwards for feeding mosquitoes on various sites (Table VII). Photograph by D. Wilson.

FIG. 2B.—Enlargement of plucked area of skin on rabbit shown in Fig. 2A. The jet black areas are patches of black hair; the scattered grey areas of different sizes are the papules of the generalized rash.



2a

2b

on the 10th day and continued until the 14th day, after which there was only a slight rise. Complement-fixing antibody appeared in both rabbits on the 11th day and continued to rise until a very high titre was attained by the 28th day.

The fibroma-vaccinated rabbit showed an accelerated antibody response. Complement-fixing antigen never appeared in the serum, but anticomplementary activity appeared suddenly on the 5th day and was present thereafter, falling slightly at the 28th day. There was a residual level of about 70 per cent reduction in pock count on the egg membrane and 40 in complement-fixing antibody, due to the fibroma inoculation 8 months earlier. A sudden rise in antibody titre, tested by both techniques, occurred on the 6th day, and thereafter the titres rose to very high levels. The highest titre of complement-fixing antibody was not reached until the end of the 3rd week.

The distribution of virus in the blood.

The experiments just described confirmed previous reports (Hobbs, 1928; Rivers and Ward, 1937) that although soluble antigen may appear to a high titre in the serum, virus particles are associated with the particulate components of the blood and do not occur free in the plasma. An attempt was made to determine what cellular elements contained the bulk of the circulating virus. A rabbit which had been inoculated subcutaneously in several sites with large doses of myxoma virus was exsanguinated on the 5th day, half the blood being mixed with heparin and half defibrinated by stirring it with roughened glass rods. Various fractionation procedures were then carried out and the final products examined for virus by titration in eggs or rabbits, and for cellular composition by total counts of the erythrocytes and leucocytes, and the examination of smears stained with Leishman's stain.

The particulate elements were separated from the plasma by centrifugation at 2° for $\frac{1}{2}$ hr. at 2000*g* and the operation was repeated once with the plasma, the final supernatant then being tested for its virus content. The cellular sediment was washed once with saline, deposited in the centrifuge, and re-suspended to the original blood volume before determination of its cellular and virus content. One sample of heparinized blood was then repeatedly centrifuged, the leucocyte-rich buffy layer being separated from the erythrocyte sediment. Owing to a technical error the final product of the concentrated buffy coat was discarded, and tests could therefore be made only of the lowest layer from which the majority of the leucocytes, and the platelets, had been separated. Another sample of heparinized blood was mixed with fibrinogen and the sedimentation of the erythrocytes accelerated, as described by Minor and Burnett (1948). This procedure was repeated once, and the supernatant fluid, containing most of the leucocytes and platelets and few erythrocytes, was examined. Results are shown in Table V.

In order to appreciate the association of virus titres and the concentrations of different particulate components of the blood, the results have been expressed as the percentage of cells of the various types present in the different samples compared with the original specimen of heparinized blood and the percentage of virus. As in previous experiments, free virus could not be demonstrated in the plasma. Procedures which eliminated platelets or erythrocytes had a negligible effect (considering the relative inaccuracy of the virus titrations) on the virus

TABLE V.—*Cellular Composition and Virus Content of Specimens of Blood Subjected to Various Treatments.*

Preparation.	Erythrocyte count (per c.mm.)	Leucocyte count (per c.mm.).	Differential leucocyte count (per cent). Granulocytes.	Lymphocytes.	Monocytes.	Platelets.	Virus titre ($\log_{10}$ I.D.$_{50}$ per ml.).
Heparinized whole blood	$6{\cdot}7\times10^6$	10×10^3	31	62	7	+++	4·5
Defibrinated blood	$6{\cdot}4\times10^6$	$2{\cdot}4\times10^3$	4	89	7	0	4·3
Plasma	0	0	0	0	0	0	0
Washed cells	$5{\cdot}4\times10^6$	$5{\cdot}9\times10^3$	35	57	8	+++	4·3
Sediment after repeated centrifugation	$3{\cdot}1\times10^6$	3×10^3	..	100*	..	0	<1·0
Supernatant after accelerated sedimentation	$2{\cdot}9\times10^6$	$4{\cdot}2\times10^3$	12	74	14	+++	4·6

* Insufficient leucocytes present in smear to enable count to be made—all those seen were lymphocytes.

TABLE VI.—*Percentages of Erythrocytes, Leucocytes and Platelets, and of Virus, in Specimens of Blood Subjected to Various Treatments.*

Preparation.	Erythrocytes.	Granulocytes.	Lymphocytes and monocytes.	Platelets.	Virus.
Heparinized blood	100	100	100	100	100
Defibrinated blood	95	3	34	0	95
Sediment after repeated centrifugation	46	0	4	0	<2
Supernatant after accelerated sedimentation	0·4	17	46	100	100

concentration, whereas removal of the leucocytes caused a great fall in the virus titre. It appeared that the virus was probably associated with lymphocytes rather than granulocytes, for a considerable fall in the percentage of granulocytes, caused by defibrination, had a negligible effect on the virus titre.

Infectivity for Mosquitoes of the Secondary Skin Lesions in Advanced Cases of Myxomatosis.

The importance of the secondary skin lesions and the lesions of the face and ears, as sources of virus which might contaminate the mouthparts of mosquitoes and render them infective, was investigated by allowing *Aëdes aegypti* to bite in these sites, and subsequently determining their infectivity for other rabbits. A rabbit was inoculated intradermally in the left flank with 20 I.D.$_{50}$ of myxoma virus. Eight days later, when the disease was far advanced (see Fig. 2), female mosquitoes which had been starved for 24 hr. were given the opportunity to bite at various sites. Each mosquito was kept in a small numbered vial. About 20 mosquitoes bit through each of the following sites: the primary lesion, at the inoculation site, the thickened skin of the upper eyelid, secondary lesions on the body, and the thickened base of the ears. Each mosquito was given an oppor-

tunity to bite a marked site on the back of a normal rabbit on the day after the "acquisition" feed: 68 of the 73 mosquitoes fed on this occasion. The results are shown in Table VII.

TABLE VII.—*Infectivity of Mosquitoes which had Bitten through Skin Lesions in Various Parts of a Rabbit with Advanced Myxomatosis.*

Site of acquisition feed.	Results. Proportion positive.	Percentage positive.
Primary lesion	11/11	100 per cent
Swollen eyelids	14/18	78 „
Swollen base of ears . . .	11/19	58 „
Secondary skin lesions on flank .	3/20	15 „

Tests were made 1 day after the "acquisition" feed.

As was expected, all mosquitoes which probed through the large primary lesion at the inoculation site became infective, and there is reason to believe that the advanced secondary tumours which sometimes occur would also be rich sources of virus. More important, from the point of view of the epidemiology of myxomatosis, is the high proportion of positive results recorded when mosquitoes bit the eyelids or base of the ears of a rabbit in an advanced stage of the disease. Under natural conditions it is probable that these lesions, which are invariably present in rabbits suffering from advanced myxomatosis, are the most important source of virus for mosquitoes. The smaller papules of the secondary rash yielded a smaller proportion of infective bites; under natural conditions they would also be less accessible to mosquitoes than the facial lesions.

DISCUSSION.

The results reported in this paper illustrate the general validity of the hypothesis outlined earlier concerning the mechanism of infection in generalized infectious diseases (Fenner, 1950). Myxomatosis in the European rabbit closely resembles mousepox in the laboratory mouse, in the occurrence of a stepwise infection of the primary lesion, the regional lymph node, generalization through the blood stream and later localization in certain areas (skin, testis) with the development of a "secondary rash". It differs from mousepox in the simultaneous appearance of virus in the circulating blood and a number of internal organs. Hurst's (1937*a*) histological studies provide an explanation of this. He reported the existence of distinctive lesions of the small arterioles in all organs of the body. The vascular tree may thus function as the "internal focus" of multiplication, analogous to the spleen and liver in mousepox, and after multiplication there deposition may occur in epithelial cells of the skin, in the testis, and elsewhere. The time intervals between the demonstration of virus at the site of inoculation, in the regional lymph node and in the viscera depend upon the presence of infective virus in the tissues examined. If there is an early non-infective stage in the growth of myxoma virus, as there is in influenza virus (Hoyle, 1948; Henle, 1949), and current experiments with myxoma virus on the chorio-allantois suggest that there is, the absolute interval between deposition of virus in the skin and its presence in the local lymph node would be shorter

than our results suggest. Further, an interval such as we have found can be demonstrated only if very small infective doses are used, so that local multiplication must occur before sufficient virus is available to be detected in the lymph node, etc. If large infective doses are used virus will be found in the local lymph node within a few minutes of the inoculation (McMaster and Hudack, 1935), but our aim has been to imitate as closely as possible the course of events which occurs in the naturally transmitted disease.

It is apparent from the final experiment that the facial lesions, an invariable development in the advanced stages of myxomatosis and a site easily accessible to mosquitoes, probably provide the principal source of virus available for the contamination of the mouth parts of insect vectors under field conditions. The primary lesion, and the secondary skin lesions on the body if they were well developed and relatively free of hair, would constitute another important source of virus.

These experiments provide additional evidence of the unsuitable nature of the blood as a source of virus for mosquitoes, if transmission is, as we believe, mechanical in nature. The plasma is free of virus, all the circulating virus being associated with particulate elements in the blood ; principally, it appears, with the lymphocytes. The situation is similar to that described in vaccinia (Smith, 1929), rinderpest (Daubney, 1928) and fowl plague (Todd, 1928). Although the plasma is usually free of virus elementary bodies, it contains a high concentration of soluble antigen in the later stages of infection with virulent myxoma virus.

The serological response in infections with myxoma virus closely parallels that occurring in other generalized virus diseases. The first indication of the presence of antibody in the serum was usually the appearance of anticomplementary activity. Donnelley (1951) discussed the significance of the anticomplementary activity of extracts of mouse lungs in the early stages of experimental influenza. She found that the rise of anticomplementary activity preceded the appearance of free antibody in the serum, and when free antibody appeared the degree of anticomplementary activity declined. The same was true of neuromyxoma when daily tests were made on the serum. With virulent myxoma virus the animals never lived long enough to permit the demonstration of free antibody in the serum, but there was a sharp rise in anticomplementary activity about the 6th or 7th day. In the rabbits infected with neuromyxoma virus neutralizing antibody appeared 1 day before complement-fixing antibody, and both rose rather slowly to reach a high titre. As free antibody was not detected until the 11th day, whereas the rabbits with myxomatosis had all died on the 9th day, it is not surprising that free antibody was not detected in the latter animals. The persistence of these antibodies in rabbits which had recovered from myxomatosis has been described earlier (Fenner, Marshall and Woodroofe, 1953).

SUMMARY.

Myxomatosis in the European rabbit is a generalized disease, in which the virus spreads in a stepwise fashion from the site of inoculation, through the local lymph node to the circulation. After a period of multiplication in an internal focus, which may be the walls of the small arterioles, secondary dissemination occurs in the skin and elsewhere. Multiplication of this secondarily distributed virus causes the development of generalized lesions of the skin, consisting of

scattered small papules over the body and generalized thickening of the face and the loose tissues in the anogenital region.

The primary and secondary skin lesions, especially the thickened tissues around the eyes and the base of the ears, as well as the papules on the body, were shown to be rich sources of virus, in terms of the ability of mosquitoes to acquire infection by biting through such lesions.

The virus particles which circulate during the viraemic stage of the disease are associated with the leucocytes, probably with the lymphocytes, and in the late stages of the disease soluble antigen appears at high titre in the serum.

The serological response was followed in rabbits infected with neuromyxoma virus. Soluble antigen did not appear in the serum in infections with this strain, but the serum became anticomplementary on about the seventh day, and on the 11th day free antibody appeared and rose steadily to reach a high level by the 14th day.

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THE SIGNIFICANCE OF THE ACCUMULATION OF CORTISONE IN AN INFLAMED AREA.*

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EARLIER studies (Menkin, 1940, 1942) indicated that adrenal cortical extract and cortisone suppress the increased capillary permeability of acute inflammation as induced by an exudate or by leukotaxine. This suppression of increased capillary permeability caused both by adrenal cortex extract and by cortisone, verified by numerous other investigators, is specifically measured by the inability of trypan blue to accumulate in an area of inflammation from the circulating blood. Besides that dye, many unrelated substances have previously been shown to accumulate in an area of acute inflammation owing to a local increased capillary permeability (Menkin, 1940*a*, 1950). Subsequent studies have shown that this suppressing effect occurs only when an alkaline exudate is used, and that cortisone is incapable of inhibiting the escape of a dye from the blood when the exudate is acid. The increased capillary permeability in the later stages of an acute inflammation, when the exudate is acid (Menkin, 1934), is due to another factor which has been termed exudin (Menkin, 1951*a*). As mentioned above, cortisone is incapable of repressing the action of exudin on the capillary wall; but this can be achieved with corticotropin (ACTH) (Menkin, 1951*b*). ACTH can be administered intravenously in rabbits, and it will inhibit the increase in capillary permeability caused by exudin (Menkin, 1951*c*). We thus have two distinct components concerned in the mechanism of increased capillary permeability in inflammation. In the initial stages of inflammation the increased outpouring of fluid is referable to leukotaxine, while in the later stages this seems primarily due to exudin (Menkin, 1951*a*). The activity of leukotaxine on the capillary wall is suppressed by the whole adrenal cortex extract or cortisone, while that of exudin is inhibited by ACTH.

The purpose of this paper is to show that cortisone can apparently localize from the systemic circulation in an inflamed area, and once there it seems to exert its suppressing tendency on the development of the inflammatory reaction by its specific property of repressing the action of leukotaxine. The actual concentration of cortisone in the inflamed area cannot be measured, but by the foregoing biological assay, namely that the hormone tends to suppress local increased capillary permeability in inflammation as measured by the local accumulation of dye from the blood stream, and by the use of proper controls, it can be shown that cortisone injected into the circulation of animals with a concomitant inflam-

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mation apparently reduces the capacity of the exudate, taken from such an area, to allow trypan blue to penetrate from the blood into the skin of otherwise normal rabbits. This type of evidence in conjunction with previous studies on the accumulation of various unrelated substances from the circulation in an acutely inflamed area (Menkin, 1950) is taken as evidence of a local accumulation of cortisone in such an area. It is, however, acknowledged that there may be other unknown factors involved in the results obtained which may eventually necessitate some other interpretation from that given by the writer.

METHODS.

Since there are two factors in an inflammatory exudate which are concerned in the mechanism of increased capillary permeability, and since cortisone has a suppressing effect only on leukotaxine, it was important to eliminate the action of any exudin present in the exudate.

This was accomplished as follows : A pleural inflammation was induced on the right side in a rabbit by the intrapleural injection of 0·2 ml. of 5 per cent or 10 per cent croton oil in olive oil. This was followed by the very slow intravenous injection of 25–50 mg. of commercial cortisone (Merck). In a previous study (Menkin, 1942) it was shown that cortisone or Compound E *per se* can suppress the increased capillary permeability of inflammation. In a subsequent recent work it was observed that the vehicle in which commercial cortisone (Merck) is suspended has, on the contrary, only the capacity of increasing capillary permeability (Menkin, 1951*d*). Therefore commercial cortisone could be used in the present study to demonstrate solely the restraining influence of the hormone *per se* on capillary permeability.

On the following day, or after several daily intravenous injections of cortisone, the exudate, if present, was withdrawn from the pleural cavity, and 0·5 ml. was injected into the abdominal skin of a rabbit, which had received a few minutes previously 30–40 mg. of corticotropin (Armour) in 3 or 4 ml. saline intravenously. The purpose of the injection of the ACTH was to obliterate any local increased passage of dye caused by exudin (Menkin, 1951*b*, *c*). In control experiments saline was substituted for cortisone in the rabbit providing the exudate and for ACTH in the rabbit receiving it.

The possibility existed that cortisone might by some unexplained mechanism inhibit the formation of leukotaxine by the injured cell without even accumulating in the inflamed area. To rule this out, a few experiments were performed in which an inflammation was induced previously. After 24 hours or so the injections of cortisone were started. In this way the formation of leukotaxine could not have been suppressed in the early stages of the inflammatory reaction. Whatever cortisone accumulated in the inflamed area directly affected the activity of any preformed leukotaxine.

To determine whether the effect obtained could be obtained in a different species with a different type of irritant, the studies on the accumulation of cortisone in an inflamed area were likewise done on the dog. The animal, under nembutal anaesthesia, received an intrapleural injection of 1·5–2 ml. of turpentine. On the following day a thoracentesis yielded exudate in many cases. Following withdrawal of this, the dog was injected slowly and intravascularly with 37 mg. of cortisone. On the next day a sample of exudate was withdrawn, and a second injection of 37 mg. of cortisone was given. This procedure was repeated daily for 3 or 4 days. The exudative samples obtained before the injection of cortisone and those following the successive daily injections of the cortisone were all introduced into the abdominal skin of 2 rabbits. One of these animals was injected with 3–4 ml. saline intravenously, and the other was treated a few minutes previously with 30–40 mg. of ACTH in the same volume. The subsequent intravenous injection of 6 ml. 1 per cent trypan blue in saline served as an indicator, as the dye accumulated locally, for determining the degree of tissue staining in the various areas treated.

RESULTS.

When the pleural exudate from a cortisone-treated rabbit is injected intracutaneously into the skin of another rabbit, in turn injected a few minutes previously with ACTH intravenously, it is found that there is distinctly less local

accumulation of dye in the injected area than in that injected with exudate from a control rabbit. The data of such experiments are presented in Table I. The suppressing effect on increased capillary permeability seems to be referable to the cortisone present in the exudate and not to the ACTH injected. This is quite evident when it is seen that the exudate from the non-cortisone-treated rabbit induces a greater increase in capillary permeability notwithstanding the injection of ACTH in the tested rabbit (Table I).

The intravenous injection of ACTH in the rabbit to be tested is essential if the masking effect of any additional exudin present in the exudate is to be nullified. This is quite clear from the observations in Table II. In the tested rabbits in this group of experiments no ACTH was previously injected, but only saline. The local staining of tissue from the exudate of the cortisone-treated rabbit is only slightly less than that of the control. Therefore it is essential first to do away with the effect of exudin in increasing capillary permeability before the suppressing tendency of any cortisone which may have accumulated at the site of inflammation can be ascertained.

Does cortisone actually accumulate in the area of inflammation, or does it inhibit the formation of leukotaxine by injured cells at the site of inflammation?

EXPLANATION OF PLATES.

FIG. 1.—Skin of abdomen of two rabbits (R. 43–39 and R. 43–40). Rabbit 43-39 received 32 mg. ACTH in 4 ml. saline intravenously several min. before intracutaneous injection in area 1 of 0·5 ml. exudate from the pleural cavity of another rabbit, which had received 4 intravenous injections of cortisone (total 115 mg.). In area 2, 0·5 ml. exudate from the pleura of an untreated control rabbit was injected. Following these intracutaneous injections of exudate 6 ml. 1 per cent trypan blue was injected intravenously, and the accumulation of dye in the area was observed. Rabbit 43–40 was given saline in place of ACTH. The cortisone-containing exudate in area 3 is much less stained than the control area 4. Evidently the cortisone-containing exudate in area 3 has to a great extent suppressed the increase in capillary permeability, as gauged by the passage of trypan blue in the area when compared to area 4. The suppressing effect is very pronounced in Rabbit 43–39 owing to the neutralization of exudin by ACTH.

FIG. 2.—Staining with trypan blue (6 ml. 1 per cent solution intravenously) in skin areas of rabbit 20–03, given 17 mg. ACTH and intravenous injections of dog exudate as follows (the earlier ones having been refrigerated).

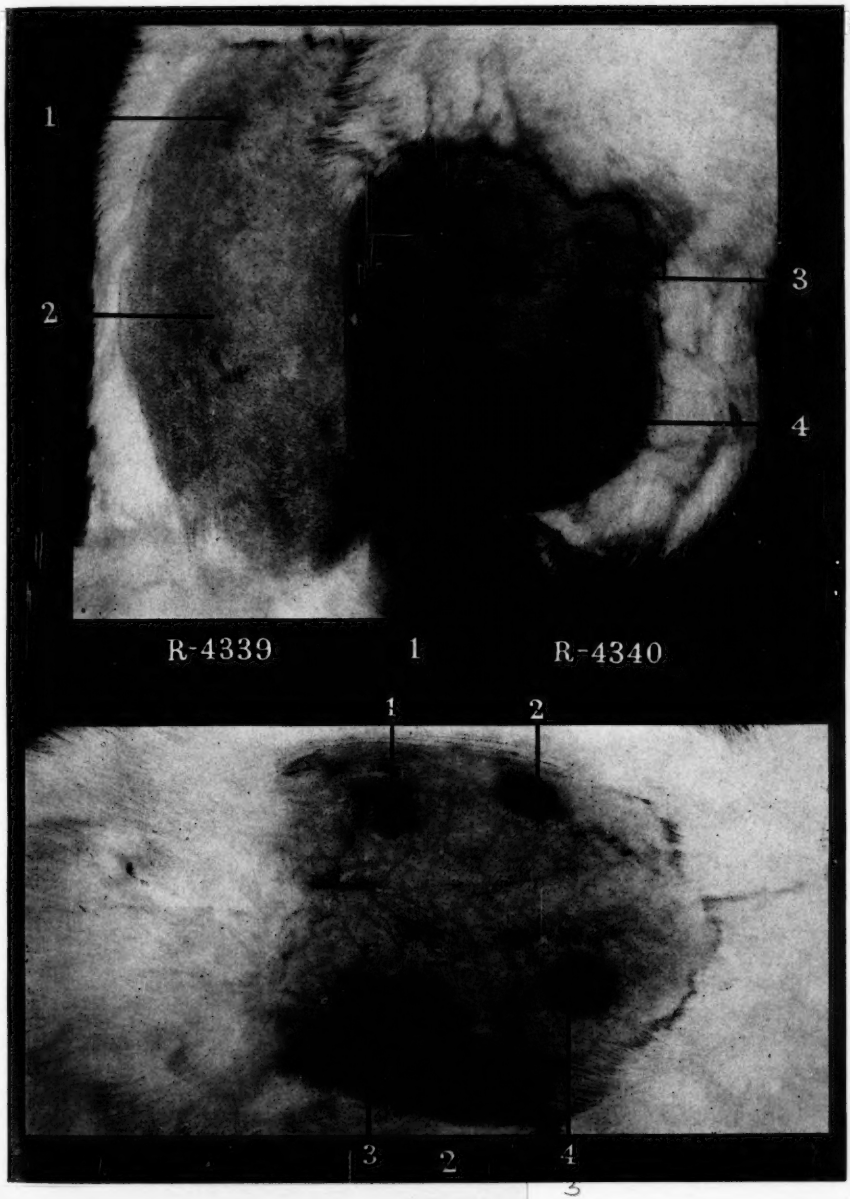
Area: 1: Pleural exudate from dog 24 hr. after intrapleural injection of 2 ml. turpentine.

Areas 2, 3 and 4: Pleural exudate from same dog on successive days following daily intravenous injection of 37 mg. cortisone.

It is clear that after three injections of cortisone in the dog there is very little accumulation of trypan blue in the skin area injected with the exudate (*cf.* areas 1 and 4). The progressive accumulation of the hormone at the site of inflammation in the dog has almost completely suppressed the increase in capillary permeability to trypan blue in the ACTH-treated rabbit.

FIG. 3.—Section of a small blood-vessel in Rabbit 43–41 taken from the skin of the abdomen 90 min. after injection of 0·5 ml. of exudate derived from the pleural cavity of a control or an untreated rabbit. ACTH (30 mg.) had been given 12 min. before injection of the exudate. Although ACTH may have had some reducing effect on the chemotactic capacity of the leukotaxine present in the exudate, local migration of leucocytes through the endothelial wall and in the extra-capillary spaces is definitely present. × 310.

FIG. 4.—Microscopic section of a small blood-vessel in Rabbit 43–41 taken from the skin of the abdomen 90 min. after injection of 0·5 ml. of exudate from the pleural cavity of a rabbit which had received 6 daily intravenous injections of cortisone totalling 165 mg. Not only has cortisone suppressed the increased capillary permeability to trypan blue, but it has likewise suppressed any diapedesis of leucocytes through the capillary wall. About 30 mg. of ACTH in 3 ml. saline was injected intravenously 6 min. before injection of the exudate. That the absence of any chemotactic response is not primarily due to the preliminary administration of ACTH is proved by the fact (Fig. 3, same animal) that here in spite of the ACTH injection, migration of leucocytes has occurred. × 310.



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In Fig. 2, area No. 1 should be labelled No. 2; area No. 2 should be labelled No. 1; area No. 4 should be labelled No. 3; and area No. 3 should be No. 4.

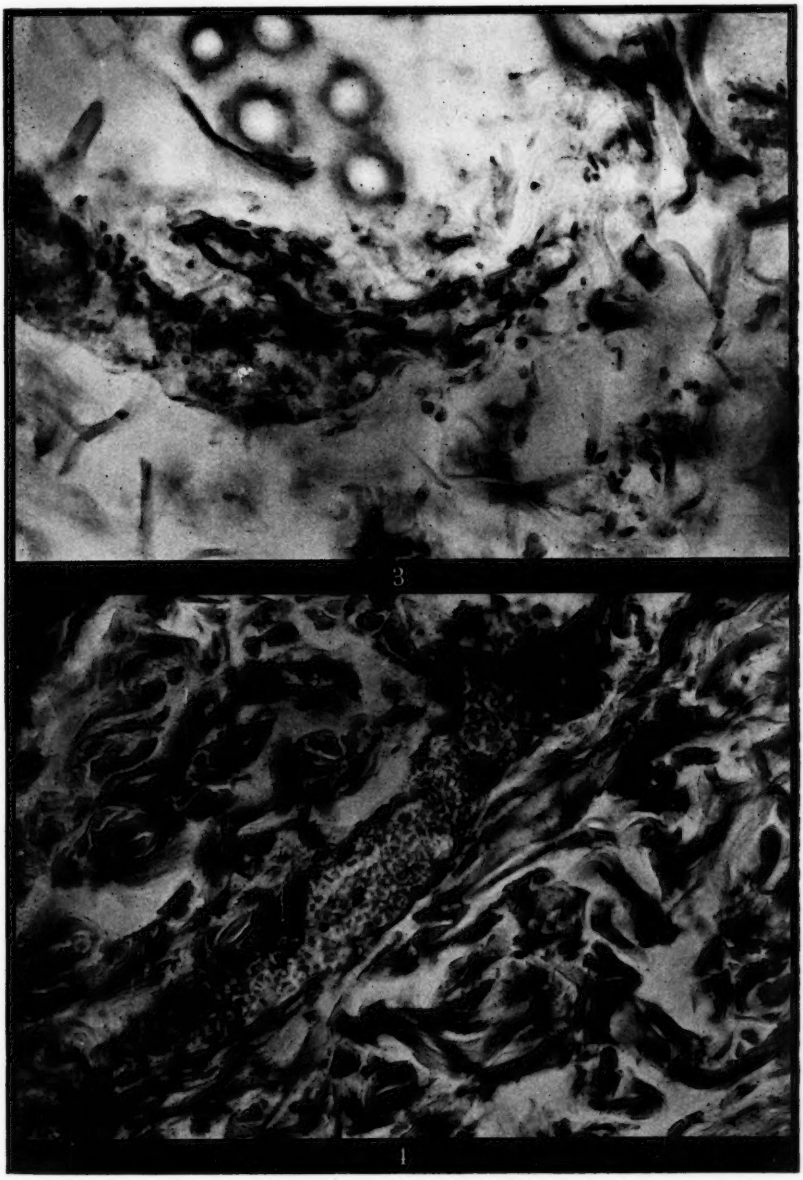

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TABLE I.—*Degrees of Staining by Trypan Blue in Skin Areas Injected with Exudate from Another Rabbit.*

Rabbit No.	Cortisone-containing exudate.	Exudate.
37–98	0	++
43–01	Trace at periphery	++
43–03	Trace	+ to ++
43–05	0*	+ to ++
43–07	0	+
,,	+*	+

All rabbits were given ACTH intravenously before injection of exudate.

* Only one intravenous injection of 50 mg. of cortisone in the rabbit from which exudate derived; in all other experiments 2 intravenous injections of cortisone totalling 100 mg. were injected.

TABLE II.—*Degrees of Staining by Trypan Blue in Skin Areas Injected with Exudate from Another Rabbit.*

Rabbit No.	Cortisone-containing exudate.	Exudate.
37–99	+++	++++
43–02	++	++(+)
43–04*	++(+)	+++
43–06†	..	++
43–08†	+	+++

All rabbits were given saline intravenously before injection of exudate in place of ACTH.

* Only 2 ml. saline injected intravenously; all the other rabbits received 4 ml.

† Only one injection of 50 mg. cortisone into the rabbit from which exudate derived; all other rabbits received two 50 mg. intravenous injections of cortisone.

The second possibility seemed unlikely, but nevertheless it was not precluded. To rule this out a pleural inflammation, as described above, was first induced in rabbits. When such a reaction had been fully established for about 24 hours, daily cortisone injections in slowly administered intravenous doses of 25–50 mg. were started. After several injections the exudate was withdrawn and assayed in the skin of a rabbit previously injected with ACTH as described previously. The exudate derived from a non-cortisone-treated animal served as control. The results are given in Table III. It is quite clear that the apparent accumulation

TABLE III.—*Degrees of Staining by Trypan Blue in Skin Areas Injected with Exudate from Rabbits Treated with Cortisone after Exudate Formation.*

Rabbit No.	Cortisone-containing exudate.	Exudate.
43–31	0	++
43–34	0*	+(+)
	0†	..
43–37‡	0	Trace
		++
43–39‡	+	++

All rabbits given ACTH before injection of exudate.

* Trace of dye at only one spot of the involved area.

† 90 mg. cortisone administered in repeated daily doses of 25–50 mg.

‡ 115 mg. cortisone administered in repeated daily doses of 25–50 mg.; in other experiments a total of 70 mg. cortisone administered.

of cortisone from the systemic circulation suppressed the activity of leukotaxine even though the inflammatory reaction had already been fully established. When compared with the effect of a control exudate, the presumably cortisone-contain-

ing exudate scarcely increased the permeability of the capillaries. When saline was substituted for ACTH, the effect of exudin present in the exudate masked the trypan blue test, and the results were not as striking (Table IV).

TABLE IV.—*Degrees of Staining in Skin Areas Injected with Exudate from Rabbits Treated with Cortisone after Exudate Formation.*

Rabbit No.	Cortisone-containing exudate.	Exudate.
43–32	++	+(+)
43–35	++(+)	++(+)
	+	..
43–38	+	++++
		Trace to +
43–40	+++	+++++*

All rabbits given saline in place of ACTH before injection of exudate.

* Peripheral flare or spread of dye in the involved area.

Fig. 1 illustrates such an experiment. The ACTH-treated rabbit (43–39) barely shows any local accumulation of trypan blue, whereas the control rabbit (43–40) displays prominent local tissue staining. Nevertheless, even in this animal one sees that the cortisone-containing exudate induces a definitely weaker reaction than the exudate from a non-cortisone injected rabbit (*cf.* areas 3 and 4).

Histological examination reveals that more than 1½ hr. after the injection of an exudate from a control non-cortisone-injected animal in an ACTH-treated rabbit, diapedesis of leucocytes is present (Fig. 3). At most the ACTH reduced, but failed to abolish completely, the chemotactic effect of the leukotaxine present in the exudate sample studied. In contrast, an exudate from a rabbit that had received 6 daily intravenous injections of cortisone (totalling 165 mg.) was injected intracutaneously in the same ACTH-treated rabbit (dose of ACTH being 30 mg.); not only was there failure of accumulation of trypan blue in the treated skin area, but there was likewise no chemotactic response (Fig. 4). It therefore seems as if cortisone accumulates from the circulating blood in an area of inflammation, and once there exerts a suppressing effect on both the local increased capillary permeability and the migration of leucocytes in the acutely inflamed area.

As mentioned above, similar experiments were done in dogs, in order to determine whether cortisone would likewise accumulate from the circulation in an inflamed area of a different species with the use of a different type of irritant. The first group of results is listed in Table V. Precisely the same technique was employed as described for the rabbit exudate. The exudate samples tested on the ACTH-injected rabbit were taken after 2 or 3 intracardiac injections each of 37 mg. of cortisone. An examination of the data reveals that only about 50 per cent of exudate samples seemed to contain cortisone, which in turn suppressed increased capillary permeability to trypan blue in the ACTH-treated rabbit. The suppressing effect of cortisone on capillary permeability in canine exudate, with the dose of cortisone employed, is less striking than that found in the rabbit (*cf.* Tables I and V). Here again preliminary injection with ACTH in the tested rabbit gives somewhat more definite results than when only saline is injected. The data of the latter group appear in Table VI. It is clear that only about one-third of the rabbits used show that the canine exudates utilized possibly seem to contain cortisone as a suppressing factor of increased capillary permeability. To determine whether successive injections of cortisone in the dog with inflammation

TABLE V.—*Degrees of Staining by Trypan Blue in Skin Areas of Rabbits Injected with Exudate from an Established Inflammation in Dogs, and first given ACTH Intravenously.*

Rabbit No.	Cortisone-containing exudate.	Exudate.
43–43	0 in centre, + at periphery	++
43–45	+++	+++
43–49	0	+
43–52	++	++
43–59	0	+++
43–64	0	++
43–65	Trace–+	+++
43–67	+ at periphery	Trace–+
43–69	0	Trace
43–76	Faint trace	,,
43–77	++	+-++
43–82	0	0
43–84	+	+

TABLE VI.—*Degrees of Staining by Trypan Blue in Skin Areas of Rabbits Injected with Exudate from an Established Inflammation in Dogs, and first given Saline in place of ACTH.*

Rabbit No.	Cortisone-containing exudate.	Exudate.
43-44	++++	++++
43–46	++(+)	++(+)
43–50	++++	++++
43–51	+++	+++++
43–61	+	+++
43–63	+ (mostly at periphery)	+++++
43–66	+(+)	++++
43–68	++++	++++
43–70	++	++
43–75	+++	+++
43–78	++(+)	+++
43–83	++	++

TABLE VII.—*The Suppression in ACTH-treated Rabbit of Increased Capillary Permeability by a Canine Exudate Following Increasing Number of Intravascular Cortisone Injections in a Dog.*

Dog No. from which exudate derived.	Tested rabbit No.	Exudate from dog when no cortisone injected. Accumulation of trypan blue.	Accumulation of trypan blue in cutaneous areas of rabbits treated with cortisone-containing exudate after intravascular injections in a dog of 37 mg. cortisone per injection.		
			1st injection.	2nd injection.	3rd injection.
224–T	43–86	++	+++	0 in centre ++ at periphery	0 (one small area has +)
	43–89	+(+)	+(+)	Trace–+ at periphery	0
y–T	20–01	+	+	+(+)	Trace
	20–03	++	++	++	0
	20–05	+++	+++	+++	+ at only one pole
191–T	20–08	+++	Trace	+	Faint trace
	20–10	+++	+ (Uneven staining)	+ (Uneven staining)	Trace to + (uneven staining)

TABLE VIII.—*The Suppression in a Saline Control Rabbit of Increased Capillary Permeability by a Canine Exudate Following Increasing Number of Intravascular Cortisone Injections in a Dog.*

Dog No. from which exudate derived.	Tested rabbit No.	Exudate from dog when no cortisone injected. Accumulation of trypan blue	Accumulation of trypan blue in cutaneous areas of rabbits treated with cortisone-containing exudate after intravascular injections in a dog of 37 mg. cortisone per injection.		
			1st injection.	2nd injection.	3rd injection.
224–T	43–88	++ at periphery	++	+++	++ (with spreading flare)
	43–90	+++	+++	++(+) (mostly at periphery)	++ (at periphery)
y–T	20–02	++++	++++	++++	+(+)
	20–04	++	++	++	++
191–T	20–09	++++	++ (in centre of area)	++	++ (in centre of area)
	20–11	+++	+++	++(+)	++

produce an accumulation of the hormone in the exudate, experiments were made with exudate before and after successive daily injections. The results appear in Table VII. It is evident that after three injections of cortisone there is considerable suppression of increased capillary permeability in the ACTH-treated rabbit (*cf.* columns 3 and 6 in Table VII). Such an experiment is illustrated in Fig. 2. The use of an ACTH-injected rabbit is again shown to be essential to illustrate the effect (Table VIII).

DISCUSSION.

The present observations indicate that intravenous cortisone seems to accumulate in a focus of acute inflammation, and once there it appears to exert a suppressing action on both increased capillary permeability and leucocytic migration. In other words, the cortisone which has lodged in the inflamed area apparently suppresses some important sequences in the development of inflammation.

What is the exact rôle of cortisone in an inflammatory reaction? John Hunter in the eighteenth century was the first to give us the concept of inflammation as a defence mechanism. This idea was more or less relegated to the background by later workers, especially under the French influence of Broussais (1816) and Gendrin (1826). Inflammation was viewed by these authors as an injurious reaction which had to be eliminated. More recently authors, including Opie (1929) and the writer (Menkin, 1931, 1940*b*, 1950), have stressed the significance of inflammation as an immunological mechansim of considerable importance in bodily economy. Menkin (1940*b*, 1942) pointed out the suppressing effect of adrenal hormones on the increased capillary permeability of inflammation. This effect has received considerable clinical application since the important work of Hench, Kendall, Slocumb and Polley (1949) on arthritis. For instance, in eye infections in which the reparative reaction may lead to opacities of the cornea and consequent blindness, the use of cortisone to abort the inflammatory reaction is doubtless of great benefit. In some arthritic disorders the hormone has been shown to be valuable (Hench, *et al.*, 1949). It is to be remembered that in these conditions the etiological agent is unknown. However, in many infections where the agent is

known, it is questionable whether cortisone is of any value (Thomas, 1952). By suppressing the inflammatory reaction, cortisone allows some infectious agents to multiply freely. The very defence mechanism which is of aid in disposing of the viable irritant is eliminated by the presence of cortisone in the inflamed area. It is quite conceivable that the specific finding of Lurie, Zappasodi, Dannenberg and Swartz (1951) and of others that cortisone is harmful in tuberculosis may have as its explanation the suppression of the inflammatory reaction. In fact, Lurie found that the administration of cortisone prevents the spread of the tubercle bacilli from the site of inoculation, probably by the suppressing activity of cortisone on capillary permability. Yet the hormone allowed a rapid multiplication of the bacilli within phagocytes. Something has been suppressed by cortisone in the development of the tubercle which then permits free multiplication of the bacilli. The observation by Shwartzman and Fisher (1952) on the free development of the poliomyelitis virus in infected mice treated with cortisone may perhaps have a similar explanation.

SUMMARY.

Cortisone injected intravenously seems to enter a focus of acute inflammation. Its presence in an exudate in a cortisone-treated animal has been demonstrated by injecting the exudate into a rabbit pre-treated with ACTH in order to eliminate the effect of exudin on capillary permeability. Cortisone in an inflamed area exerts a suppressing action on the inflammatory reaction. This is presumably accomplished by inhibiting the action of leukotaxine in increasing capillary permeability and in inducing leucocytic migration. This effect is not due to inhibition of leukotaxine formation.

The accumulation of cortisone in an inflamed area and its suppressing effect on the inflammatory reaction explains its beneficial effect in inflammatory processes such as rheumatoid arthritis and infections of the eye. This suppressive effect may be disadvantageous in tuberculosis and other conditions.

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ON THE LOCALIZATION OF CORTICOTROPIN (ACTH) IN AN INFLAMED AREA.*

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IN the preceding paper it was shown that cortisone seems to pass from the blood into an inflamed area, and once there to exert a suppressing action on the inflammatory reaction as manifested by the local increased capillary permeability and the migration of leucocytes. Does some corticotropin (ACTH) likewise pass from the circulation into an inflamed area, and what effect has this localization on the inflammatory reaction ? It is well known that ACTH acts on the adrenal cortex ; is there also a direct effect by this hormone when some of it seeps from the blood stream into the acutely inflamed area owing to a marked local increase in capillary permeability ?

METHODS.

Dogs serve as source of exudate. They were injected intrapleurally, under sodium pentobarbital anaesthesia, with 1–2 ml. of turpentine on the right side. Several days to a few weeks later they were reinjected at the same site with the same amount of turpentine. An abundant acid exudate was frequently recovered by this double injection of the irritant (Menkin, 1940*a*). Exudin is usually recovered from such acid exudates : its property of increasing capillary permeability has been shown to be repressed by ACTH (Menkin, 1951*a*, *b*, *c*). About 24 hr. after reinjection of the irritant in the pleural cavity a sample of exudate was withdrawn and refrigerated. Thirty milligrammes of ACTH (obtained from the Armour Co.) in 3 ml. of saline were then injected into the heart. On the following day another exudative sample was removed, and again refrigerated. A second intracardiac injection of ACTH was given, and this procedure of injecting ACTH in 30-mg. doses and of withdrawing the exudate from the chest cavity was repeated for 3 or 4 days. The various samples of canine exudate were then ready to be tested on a rabbit as described in the preceding paper (Menkin, 1953), with the difference that 25 mg. of commercial cortisone (Merck) were injected intravenously just prior to the intracutaneous injections of exudate. Cortisone was given in an endeavour to suppress any leukotaxine present in the exudate which may, in turn, mask the full effect of exudin on capillary permeability (Menkin, 1951*b*). As soon as the skin injections were completed, about 6 ml. of 1 per cent trypan blue in saline was injected intravenously. The local accumulation of the dye in the treated areas served as a gauge of the local increased capillary permeability. A control received 1 ml. saline intravenously instead of cortisone. With the marked local increased capillary permeability in an inflamed area, it is reasonable to suppose that some injected ACTH would penetrate from the circulating blood into the inflamed area. Once there the ACTH would have a specific effect in suppressing the increased capillary permeability caused by exudin. Daily injections of ACTH would thus have a greater suppressing effect on exudin, as measured by the activity of the extracted exudin from the inflammatory exudate. The various exudin fractions extracted were tested in the skin of a rabbit, which was subsequently injected intravenously with trypan blue as described above. The exudin fractions were obtained as described previously (Menkin, 1951*a*). The exudate was treated with ammonium sulphate at 1/3 saturation. By centrifugalization the resulting euglobulin precipitate was discarded. The

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suspended albumin-pseudoglobulin fraction was dialyzed against tap-water until free of SO^4. This rather crude fraction has been shown to contain the factor, exudin; further purification is possible (Menkin, 1951*a*), but for this experiment unnecessary.

RESULTS.

When canine exudate, obtained first before the intravascular injection of ACTH, and then after the daily administration of this hormone, is tested in a cortisone-treated rabbit, there is a tendency for a reduction in capillary permeability as indicated by the local accumulation of trypan blue in the treated areas. The data of such experiments are assembled in Table I. This suppressing ten-

TABLE I.—*The Suppression in a Cortisone-treated Rabbit of Increased Capillary Permeability by Canine Exudate Following an Increasing Number of Intravascular ACTH Injections in a Dog.*

Dog No. from which exudate derived.	Tested rabbit No.	Exudate from dog when no ACTH injected. Accumulation of trypan blue.	Accumulation of trypan blue in cutaneous areas of rabbits treated with ACTH-containing exudate obtained from a dog following the repeated intravascular injections of 30 mg. ACTH.		
			1st injection.	2nd injection.	3rd injection.
1–91	20–14	+++	+++	..	..
,,	20–16	++++*	+(+)	+(+)	..
,,	20–19	++	+(+)	++	Trace
,,	20–22	++	..	..	+
1–56	20–31	++	+(+)	Trace	..
,,	20–33	+++	..	+++	..
76	20–45	+++	++ (small area stained)	+(+)	..
,,	20–51	++++*	..	..	++†
,,	20–53	+++*	..	..	++†
,,	20–55	+++*	..	..	Faint trace
,,	20–57	+++	..	..	+(+)

* Spreading reaction. † No spreading reaction.

dency on the part of the exudate apparently containing ACTH is seen in 9 out of 11 experiments. The effect may be detected after a single intravascular injection of ACTH; but it seems to be more definite following several daily injections of the hormone.

In the non-cortisone-injected rabbits this suppressing tendency is distinctly less visible. Only 5 out of 13 experiments show some suppressing effect. It would appear that cortisone, by obliterating the effect of leukotaxine on capillary permeability, brings out more effectively the possible suppressing tendency of ACTH on exudin present in an acid exudate (Table II). It is quite clear that whatever leukotaxine may be present, not being affected by ACTH, as regards capillary permeability, masks any effect that the hormone may have on exudin in the exudate (Menkin, 1951*b*).

There is another point which perhaps deserves attention. In several experiments (Tables I and II) the canine exudate, when introduced into the skin of rabbits, induces, besides an increase in capillary permeability, a flare or spreading effect of the dye. This is doubtless referable to the presence of hyaluronidase in the exudate. What is curious is that ACTH which has apparently penetrated into the exudate from the circulation, abolishes, not the spreading effect due to hyaluronidase, but rather the increase in capillary permeability to the dye in the

enlarged skin area. It is conceivable that the increase in capillary permeability as seen in the widespread accumulation of dye is referable to either exudin or something closely allied to it, since it is suppressed by ACTH. This, however, requires further study.

Does ACTH, which appears to penetrate the site of acute inflammation directly from the blood, not only suppress increased capillary permeability (Fig. 1) through its specific inhibiting effect on exudin, but also repress the diapedesis of polymorphonuclear leucocytes ? It is to be recalled that although ACTH does not seem to have an appreciable influence on the increase in capillary permeability induced by leukotaxine, the migration of leucocytes caused by the formation of leukotaxine at the site of inflammation is at times repressed by ACTH provided the fraction of leukotaxine is not excessively potent (Menkin, 1951*b*). In other words, it seems as if ACTH can to some extent inhibit diapedesis. Microscopic observations in the present experiments seem to indicate that as more and more ACTH presumably accumulates at a site of acute inflammation, there is a gradual suppression of chemotactic activity. After 4 intravascular injections of ACTH, there is almost complete suppression of diapedesis.

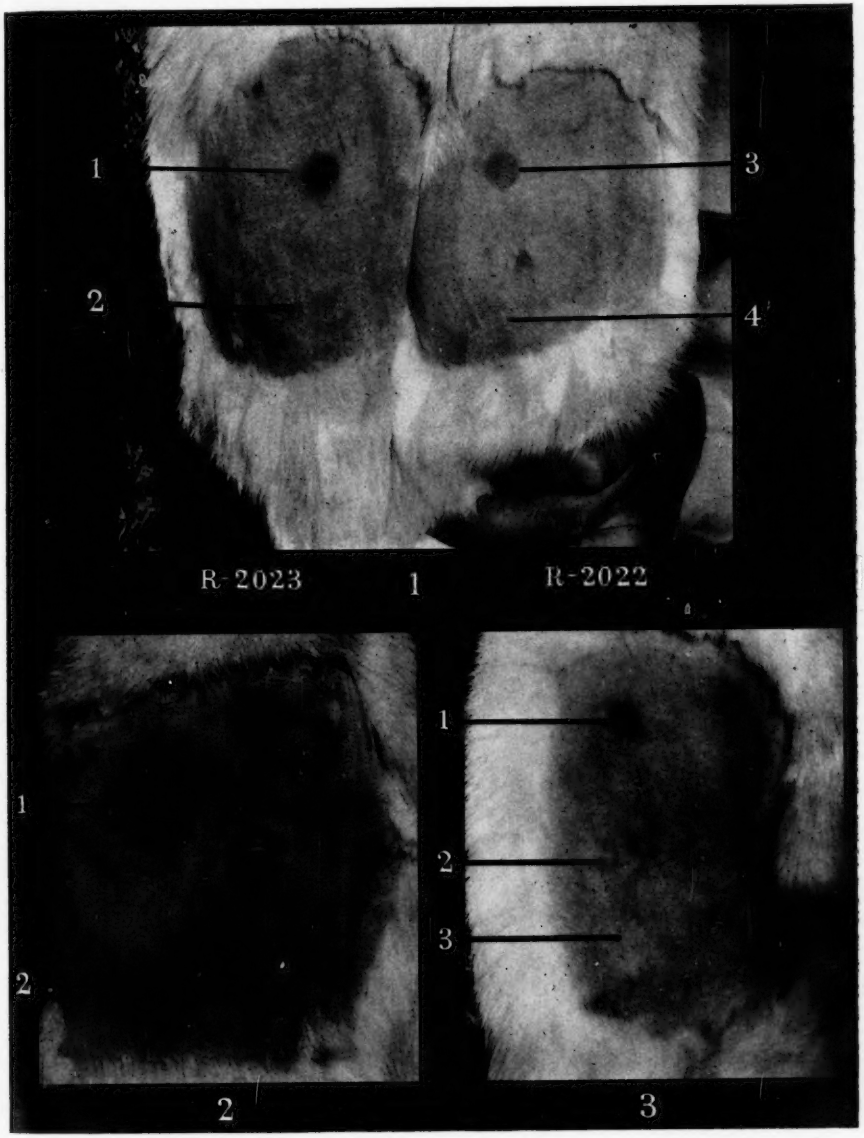
Exudin was extracted from the various samples of exudate recovered. Since ACTH has been shown to suppress the effect on capillaries caused by exudin (Menkin, 1951*b*, *c*), with the entrance of some of this hormone into the site of inflammation the increase in capillary permeability caused by exudin would

EXPLANATION OF PLATES.

FIG. 1.—Suppressing effect of ACTH in an exudate from a dog on the capillary permeability of a rabbit injected intracutaneously with such an exudate. Rabbit 20–23 received an intravenous injection of 1 ml. saline. Almost immediately afterwards, 0·5 ml. of exudate (pH 6·85) from dog 1–91, reinjected with turpentine in the pleural cavity, was injected in area 1 : 0·5 ml. of exudate from the same dog after 3 daily intravascular injections of 30 mg. ACTH was injected into the skin in area 2. Canine exudate (after ACTH) suppresses the increase in capillary permeability to trypan blue. The same procedure was followed in rabbit 20–22, except that several minutes before the intracutaneous injections of exudates the rabbit was also injected intravenously with 25 mg. cortisone. The results appear in areas 3 and 4. It is clear that in the acid exudate of a reinjected dog there is little leukotaxine but considerable exudin. The preliminary injection of cortisone seemed unnecessary in this animal. Presumably ACTH in the exudate by itself was capable of suppressing the local increased capillary permeability.

FIG. 2.—Rabbit 2061. In area 1, 0·5 ml. of canine exudate (pH 7·16) was injected, from the pleural cavity of dog 76–T ; the animal had been reinjected with turpentine. Note the marked increase in capillary permeability. In area 2 was injected 0·5 ml. of exudate (pH 7·2), obtained following 3 daily intravascular injections of 30 mg. ACTH, following reinjection of the irritant. Note that the exudate is now incapable of eliciting a significant increase in capillary permeability to trypan blue (*cf.* areas 1 and 2). The preliminary administration of cortisone in test rabbit 2061 was evidently unnecessary in order to demonstrate the suppressing effect of the exudate following repreated intravascular administration of ACTH in a dog with an inflammation in its pleural cavity.

FIG. 3.—As ACTH penetrates an area of inflammation, the effect of exudin in increasing capillary permeability is suppressed. Rabbit 20–30 was injected intracutaneously in the surface of the abdomen with exudin extracted from the exudate of dog 1–91 following reinjection of the the irritant in its pleural cavity. The exudin was diluted with physiological saline (1:1) and 0·5 ml. of the mixture was injected into the skin of R. 20–30 in area 1. Subsequently the dog received daily intravascular injections of 30 mg. ACTH. After 3 and 4 injections of ACTH the exudate was withdrawn from the pleural cavity and exudin extracted from each sample. In area 2, after 3 intravascular injections of ACTH there is hardly any increased capillary permeability induced by the recovered exudin. This suppressing effect is even more pronounced following 4 intravascular injections of ACTH. The local increase in capillary permeability to trypan blue is virtually wholly inhibited (area 3). This suppressing effect is not seen in the exudin recovered from the exudate of a dog not injected with ACTH.



Menkin.

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TABLE II.—*The Suppression in Non-cortisone-treated Rabbits of Increased Capillary Permeability by Canine Exudate following an Increasing Number of Intravascular ACTH Injections in a Dog.*

Dog No. from which exudate derived.	Tested rabbit No.	Exudate from dog when no ACTH injected. Accumulation of trypan blue.	Accumulation of trypan blue into cutaneous areas of control rabbits treated with ACTH-containing exudate obtained from a dog following the repeated intravascular injections of 30 mg. ACTH.		
			1st injection.	2nd injection.	3rd injection.
1–91	20–15	+++	+++	..	..
	20–13	+++	+++	..	..
	20–17	++++*	++++†	++++†	..
	20–18	++*	++‡	++‡	..
	20–20	+++	+++	+++	++ (small stained area)
	20–21	++*	+++	++	++
	20–23	+++	..	..	+
1–56	20–34	+++	..	+++	..
76	20–46	+	+	+	..
	20–52	++++*	..	..	++‡
	20–54	++*	..	..	+‡
	20–56	++++*	..	..	++‡
	20–58	++++*	..	..	++†

* Spreading reaction.

† Spreading reaction present but less in extent than in area treated with exudate presumably containing no ACTH.

‡ No spreading reaction.

TABLE III.—*The Reduction of the Increased Capillary Permeability Induced by Exudin in an Exudate by the Accumulation of ACTH from the Circulation in an Area of Inflammation.*

Tested rabbit No.	Exudin from exudate of dog prior to any injection of ACTH. Accumulation of trypan blue.	Accumulation of trypan blue in cutaneous area of a rabbit treated with exudin from the exudate of a dog after repeated injections of 30 mg. ACTH.		
		After 1 injection of ACTH.	After 3 injections of ACTH.	After 4 injections of ACTH.
20–24	+++	+(+)	Trace	..
20–25	++	Faint trace	0	..
20–26	++	+	Trace to +	+
20–27	++++	++	++	+
20–28	+++	..	+(+)	+
20–29	+++++	..	++++	++
20–30	+++	..	+	Trace to +

correspondingly be reduced. The data of this group of experiments are shown in Table III.

Clearly the exudin recovered from the exudate prior to the injection of ACTH is very active in inducing an increase in capillary permeability. Subsequent daily intravascular injections of ACTH tend to suppress the activity of exudin recovered from withdrawn samples of pleural exudate. This would indicate that some ACTH has quite likely entered the inflamed area and suppressed the activity of the existing exudin. (Fig. 3 is an illustration of such an experiment.)

DISCUSSION.

These observations indicate that like cortisone, ACTH injected into the blood stream passes into an area of acute inflammation. It then apparently exerts a

suppressing effect on the local increased capillary permeability caused by exudin, and to some extent, after repreated injections of the hormone, on the diapedesis of leucocytes. As a precaution against the possible effect of leukotaxine in the exudate on capillary permeability 25 mg. commercial cortisone was first injected intravenously (Table I, Fig. 1). There seems, however, to be relatively little leukotaxine in an acid exudate of the reinjected pleural cavity of a dog. In many instances it was found that the mere repeated injection of ACTH into the circulation of a dog was sufficient to enable the exudate to suppress the increase in capillary permeability to trypan blue in rabbits. Since ACTH has practically no effect on this particular property of leukotaxine (Menkin, 1951*b*), this suggests that in an acid exudate the primary component which increases capillary permeability is exudin and not leukotaxine. This is shown in Fig. 2.

Does some ACTH penetrate the inflamed area from the circulation or does it act on the adrenal cortex, with the liberation of various steroids which, in turn, induce the suppressing effects described here ? This is, of course, the physiological action of ACTH, and for that reason observations were made on rats adrenalectomized by Mr. Donald Mann. The increase in capillary permeability caused by exudin was suppressed by ACTH in the adrenalectomized as well as in the non-adrenalectomized animals. The effect of ACTH is thus a direct one without the mediation of the adrenal cortex. In addition the earlier work of the writer has indicated the great local increase in capillary permeability at the site of acute inflammation (Menkin and Menkin, 1930 ; Menkin, 1931). The capillaries in such a site are like a sieve, and there is an immediate outpouring of material from the circulation into the extra-capillary spaces. Graphite particles and even bacteria are capable of going through the altered capillary membrane. Therefore, the passage of a certain amount of ACTH into the inflamed area is not an unwarranted supposition. That the rest may go to the adrenal cortex is not questioned ; but a sufficient amount of ACTH probably enters directly the site of inflammation, where it exerts its suppressing effect on the inflammatory reaction. The indirect action of ACTH via the adrenals is a process difficult to envisage. The original experiments, which indicated the direct suppressing effect of ACTH on exudin, were done by intracutaneous injections after mixing exudin or an acid exudate with the hormone. There was an immediate suppressing effect on the increase in capillary permeability (Menkin, 1951*b*). It is well known that the intracutaneous route is a very slow path of absorption, and yet the suppressing action of ACTH was so rapid as to preclude a probable effect on the adrenals. It has been shown also that ACTH does not repress the increase in capillary permeability to trypan blue when induced by leukotaxine (Menkin, 1951*b*). Leukotaxine has recently been shown to be found in the severe traumatic injury following the crushing of muscle tissue (Menkin, 1952). Yet, the intravenous injection of ACTH fails to repress the increase in capillary permeability caused by this leukotaxine. If the main effect of ACTH is to liberate cortisone or other steroids from the adrenal cortex, some of these hormones would suppress the increased capillary permeability caused by leukotaxine (Menkin, 1940*b*). Recently Compound F has been studied in regard to this property, and found as compounds A, B and E (cortisone) of Kendall have been shown earlier, to suppress the effect of leukotaxine on capillary permeability in rabbits (Menkin, 1942). It is also to be recalled in this connection that, contrary to its ineffectiveness in altering increased capillary permeability caused by leukotaxine when introduced into the blood

stream (Menkin, 1952), ACTH intravenously injected suppresses the local effect of exudin on the permeability of the capillaries (Menkin, 1951*c*). In view of these various considerations, it appears as if some ACTH, like cortisone, gradually enters the site of inflammation from the blood stream ; and once there exerts a direct suppressing effect on the inflammatory reaction.

The clinical significance of these observations is quite obvious. It is conceivable that the beneficial effect of cortisone and of ACTH in some specific inflammatory conditions is referable to the direct accumulation of these homones at the site of inflammation where they can exert their suppressing action. It is interesting to note in regard to the direct effect of cortisone, Compound F and ACTH (Armour and Organon ACTH used) on cells, in recent studies which have been reported *in extenso* elsewhere (Menkin, 1953*b*), that these hormones, as well as desoxycorticosterone acetate, suppress to a large extent the incidence of cleavage of fertilized ova of *Arbacia punctulata*. The reaction is quite specific, for testosterone propionate (Ciba) and somatrofin (STH of Horner) fail to inhibit the normal cleavage of such ova. The vehicle of commercial cortisone likewise fails to suppress the normal incidence of cleavage. In the case of ACTH the observed fact is not referable to a protein effect. Cleavage is an expression of normal cellular activity. It would seem that some of the adrenal steroids and ACTH have a direct toxic cellular action *in vitro*. The effect is absent when a protein anabolic hormone (the steroid testosterone) or a growth hormone from the pituitary (STH or somatrofin) are used. The effect is quite specific, suggesting that perhaps the mechanism of suppression in inflammation is a direct one at a cellular level. These hormones would thus suppress important cell activity. In this connection present studies which will be reported indicate that Compound F suppresses in dogs the formation of both leukotaxine and the leucocytosis-promoting factor (LPF) at the site of inflammation. These substances appear to be formed by the injured cell (Menkin, 1950, 1953*a*).

SUMMARY.

As indicated by its suppressing effect on increased capillary permeability in an area into which an acid or neutral exudate has been injected, it appears that ACTH injected intravascularly penetrates an inflamed area, and appreciably accumulates there if the injections are repeated. It also appears to inhibit somewhat the diapedesis of leucocytes. Similar results were obtained when exudin extracted from an exudate was injected locally instead of the exudate itself.

This local and direct effect in an inflamed area, and its possible clinical significance, are discussed in the light of the further observation that ACTH and related compounds interfere with the cleavage of sea urchin ova.

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QUANTITATIVE STUDY OF THE GRANULOPECTIC ACTIVITY OF THE RETICULO-ENDOTHELIAL SYSTEM. I: THE EFFECT OF THE INGREDIENTS PRESENT IN INDIA INK AND OF SUBSTANCES AFFECTING BLOOD CLOTTING *in vivo* ON THE FATE OF CARBON PARTICLES ADMINISTERED INTRAVENOUSLY IN RATS, MICE AND RABBITS.

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To investigate the granulopectic activity of the cells which constitute the reticulo-endothelial system various authors have described methods concerned with the injection of vital dyes, or suspensions of particles such as colloidal silver, manganese, India ink, thorium (Cappell, 1929; Wislocki, 1924; Foot, 1920, 1923; Nagao, 1920, Brickner, 1927; Gordon and Katsh, 1949). When particulate matter is injected intravenously, it is the pectic activity of the phagocytes of the reticulo-endothelial system lining the vascular bed which is studied, the suspensions of particles normally used being unable to pass through the vessel walls. In most cases (except in that of thorium) the investigators have studied, by histological methods only, the distribution in the various organs of the particles which had been phagocytosed, without determining either the rate of clearance of the blood or the accumulation in the tissues quantitatively.

We have recently described a method of study of the granulopectic activity of the reticulo-endothelial system in various animal species by injection of a commercial colloidal suspension* of carbon particles of known size (about 200 to 500 Å) (Halpern, Biozzi, Mené and Benacerraf, 1951). Using quantitative methods, the rate of the clearance of carbon from the blood and its accumulation in the various organs were investigated for various doses of carbon. It was found that in rats, when doses of carbon of 16 mg./100 g. or less were injected, the clearance of carbon from the blood was practically complete in an hour and the concentration was an exponential function of the time. Most of the carbon was found in the liver and spleen (90 per cent) while the lungs showed no carbon at all. When doses of carbon above 16 mg./100 g. were injected, the rate of disappearance of carbon from the blood was much more rapid and the curve of clearance could no longer be expressed by a simple mathematical relationship. A considerable amount of carbon was retained in the lungs, kidneys and other organs. Apparently another factor determines the different behaviour of carbon when injected in doses greater than 16 mg./100 g. Doses of ink above 24 mg. of carbon/100 g. constantly caused the death of the animals within a few hours. The same phenomenon was observed in mice, but more irregularly, much less accentuated, and for much higher doses of carbon (above 32 mg./100 g.).

* Commercial India ink, Pelikan, Gunther Wagner, Hanover.

It was suggested (Biozzi, Benacerraf, Mené and Halpern, 1951) that in rats, doses above 16 mg. of carbon/100 g. caused a considerable liberation of thromboplastin which exhausted the reserves of anticoagulants in the blood; this, added to the considerable surface action of the carbon particles, caused a microcoagulation of fibrin in the blood. Analyses of the amount of fibrinogen in the blood of rats before and after the injection of the various doses of ink showed that the amount of fibrinogen was greatly depleted after the administration of doses of carbon greater than 16 mg./100 g. The same doses of carbon were also accompanied by a considerable decrease in the number of blood platelets.

In mice, in which the paradoxical action of high doses of ink was much more irregular and much less marked, the number of blood platelets was not considerably affected by these high doses of carbon. It is probable that in mice even large doses of ink do not cause a significant coagulation of fibrin.

This mechanism of microcoagulation of fibrin *in vivo* may determine the unexpected action of large doses of ink upon the rate of clearance of carbon from the blood and its distribution in various organs. It is conceivable that the microcoagulation of fibrin *in vivo* causes the formation of larger aggregates of carbon particles and fibrin and modifies in this way the rate of clearance of particles from the blood.

To substantiate this hypothesis we are submitting other evidence based upon the effect on this phenomenon of two groups of substances which either activate or inhibit the coagulation of blood.

All commercial India ink preparations contain mainly carbon particles and and an organic shellac which is a stabilizing agent for the suspension as well as an ingredient which gives lustre to the ink. To investigate whether the blood clotting and toxic effects of the commercial suspensions of carbon generally used by various authors were attributable to the carbon particles or to this shellac, we obtained from Gunther Wagner a special suspension of carbon in fish glue and investigated its properties. We are also reporting data which establish that all untoward reactions observed with high doses of India ink, such as coagulation of fibrin *in vivo*, and toxicity, are attributable to the shellac and not to the carbon particles.

MATERIALS AND METHODS.

Substances which Promote Blood Coagulation.

We have studied the action of thromboplastin (Thrombokinase Hoffman La Roche) and of thrombin (Thrombase Roussel), injected intravenously on the rate of clearance of carbon from the blood and its distribution in the various organs, after standard doses of India ink were administered intravenously. Commercial India ink "Pelikan" which contains 80 mg. carbon/ml. was used in all the experiments. The size of the carbon particles was found to be about 200 to 500 Å by the electron microscope. For animal injection 20 per cent dilutions of ink were made in normal saline containing 1 per cent gelatin, to afford the stabilizing protection of a colloidal agent. Control experiments *in vitro* have shown that within the limits of concentration used, the particles of carbon remain in stable suspension either in serum or in citrated blood. These experiments were carried out on 30 rats weighing 120–140 g. and 15 mice weighing 18–25 g. A dose of 16 mg. carbon/100 g. was given to rats and 24 mg./100 g. to mice; it has been shown that these doses cause equivalent blood concentrations of carbon in the two animal species. Injections were made into the tail vein.

The rate of clearance of carbon from the blood was investigated. Blood samples were obtained at regular time intervals by puncturing the retro-orbital venous plexus with a capillary glass pipette calibrated to 0·025 ml. and previously washed with heparin (Halpern

and Pacaud, 1951). The blood was lysed in 2 ml. of 0·1 per cent Na_2Co_3 and the amount of carbon in the blood determined electrophotometrically.

The distribution of carbon was studied quantitatively in the liver, spleen, lungs and kidneys for the same doses of ink, by completely digesting the organ in concentrated alcoholic NaOH and finally weighing the amount of carbon recovered (Halpern *et al.*, 1951).

The suspension of commercial thromboplastin (Thrombokinase Hoffman La Roche) was prepared according to the accepted technique for measuring the prothrombin time and 0·2 ml. of this solution was injected intravenously into rats. Commercial thrombin (Thrombase Roussel) was administered intravenously in a dosage of 88 mg./100 g. All the solutions were injected slowly into the tail vein 2 min. before the injection of ink. The animals withstood the injections perfectly well.

Substances which Prevent Blood Coagulation.

We have studied the effect of two anticoagulants, heparin and dicoumarol, on the rate of clearance of carbon from the blood and on its distribution in the organs for various doses of ink. This study was carried out in rats and rabbits, both of which species apparently exhibit the phenomenon of coagulation of fibrin *in vivo* following large doses of carbon (32 mg./100 g. in rats, 16 mg./100 g. in rabbits). The effect of the anticoagulants was not studied in mice because the injection of ink does not cause significant changes in blood clotting consistently.

The experiments were carried out on 45 rats weighing 110–140 g. and on 30 rabbits weighing about 2 kg. Commercial India ink "Pelikan" was used and the dilution made as described above. The doses given to rats were 8, 16 and 32 mg. carbon/100 g. Doses of ink above 16 mg. carbon /100 g. cause a severe thrombocytopenia and a significant decrease in the blood fibrinogen concentration (Biozzi *et al.*, 1951). The rate of clearance of carbon from the blood and its distribution in various organs was investigated as described above. The rats treated with heparin received 25 mg. (Liquemine Roche) into the tail vein 2 min. before the injection of India ink. The study was made on 5 animals for each dose of carbon.

In the rabbit doses of 8 and 16 mg. carbon/100 g. were injected into animals which had previously received 50 mg. heparin intravenously. Blood samples were taken at regular intervals and the amount of carbon in the blood ascertained in the same way as for rats. In the rabbit the injections of carbon were made in the ear vein and the samples of blood taken from the artery in the other ear.

Dicoumarol was given to a group of rats *per os*, 5 mg./100 g. being administered daily for 3 days by gastric cannula. The effect on blood clotting was measured by determining either the blood coagulation time on a slide or the prothrombin time. Only those animals whose blood coagulation time was greater than 8 min. (normal with our technique was 30 sec.) were used for the injection of ink. This corresponded to a prothrombin level below 5 per cent of normal; 15 animals were used in these experiments, 5 rats for each dose of ink.

Injection of a Suspension of Carbon Particles without Shellac.

A carbon suspension in fish glue was obtained from Gunther Wagner, Hanover (Ink No. C11/1431a). This ink contained approximately 100 mg. carbon/ml. suspended in a solution of fish glue. It was centrifuged at 5000 r.p.m. for 15 min. to remove all particles above 500 Å. The supernatant fluid was then analysed for carbon by weight and dilutions were made in distilled water and gelatin so as to have preparations containing either 16 mg. carbon/ml. in 1 per cent gelatin or 32 mg. carbon/ml. in 2 per cent gelatin. The solutions of gelatin were previously neutralized with ammonia. The preparation containing 16 mg. carbon/ml. was used for doses of 8 mg./100 g. and 16 mg./100 g.; the preparation containing 32 mg. carbon/ml. was used for higher doses. The suspensions were homogenous and stable in serum. They were injected into rats intravenously in the doses described. Care was taken to keep the suspensions at 37° to avoid solidification of the gelatin.

Injections of 8 mg., 16 mg. and 32 mg./100 g. were given to 40 rats. The rate of disappearance of the particles from the blood and the accumulation of carbon in the various organs were studied as previously described. The results (Fig. 9) are presented as the mean for the values obtained, and are compared with those obtained for the same doses of carbon in commercial ink containing shellac.

RESULTS.

Substances which Promote Blood Clotting.

The action of the substances which promote blood coagulation on the rate of clearance of carbon from the blood and its distribution in the various organs was investigated by injecting into rats 16 mg. of carbon/100 g. body weight and comparing the results obtained with those from control animals. It has been established that for this dose of carbon as well as for the smaller doses the curve of clearance of the blood has a simple mathematical shape and the liver and spleen are the only organs which accumulate carbon particles (Halpern *et al.*, 1951).

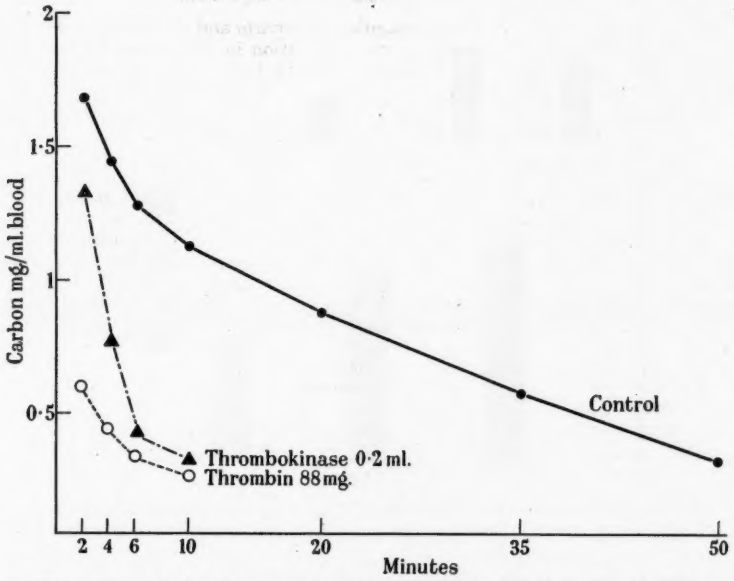


FIG. 1.—Effect of thrombin and thromboplastin (thrombokinase) on the rate of blood clearance of carbon particles in rats following intravenous injection of 16 mg. carbon/100 g. body wt. (commercial ink Pelikan).

We have shown also that this dose of carbon does not change appreciably in rats the amount of fibrinogen in the blood (Biozzi *et al.*, 1951). The results of these experiments are presented as the mean for each group of animals in Fig. 1 and 2.

Following the administration of blood clotting agents and carbon one notes that there is (*a*) much more rapid clearance of carbon from the blood and (*b*) accumulation of more carbon in the lungs than is seen when carbon is injected alone. In mice also thromboplastin causes a rapid disappearance of the carbon particles from the blood (Fig. 3).

The distribution of carbon in the organs of rats following the injection of two different doses of thromboplastin is presented in Fig. 2. In the control animals receiving 16 mg. carbon/100 g., almost all of the injected carbon is found in the liver and spleen. When a large dose of thromboplastin is administered,

considerable quantities of carbon are seen to accumulate in the lungs while the liver and spleen are found to contain less carbon. This behaviour was observed also in mice receiving a corresponding dose of thromboplastin.

The histological studies carried out on the animals treated with thromboplastin were in agreement with the quantitative analysis. In the liver the carbon

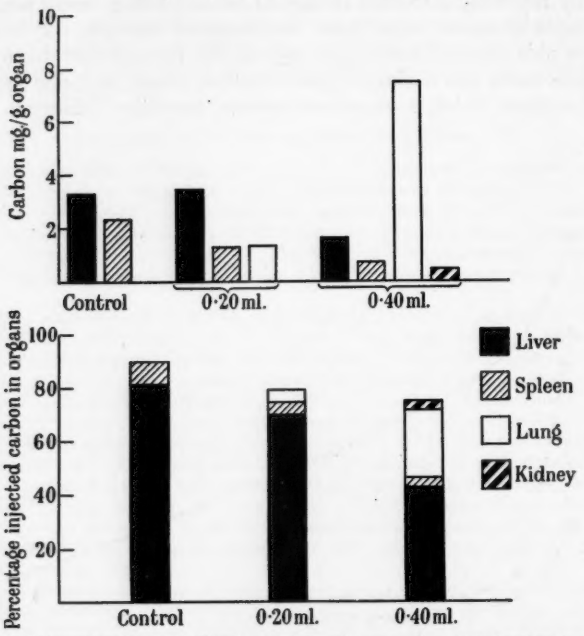


FIG. 2.—Effect of thromboplastin (0·20 ml. and 0·40 ml.) on carbon distribution in various organs of rats following intravenous injection of 16 mg./100 g. (commercial ink Pelikan).

particles were found in the Kupffer cells, more particularly in those located around the portal tracts (Fig. 10). In the control animals this special distribution was not observed (Fig. 11). In the spleen the amount of carbon in the cells appeared much smaller in the animals treated with thromboplastin than in the control animals, which is again in agreement with the results of the analysis by weight.

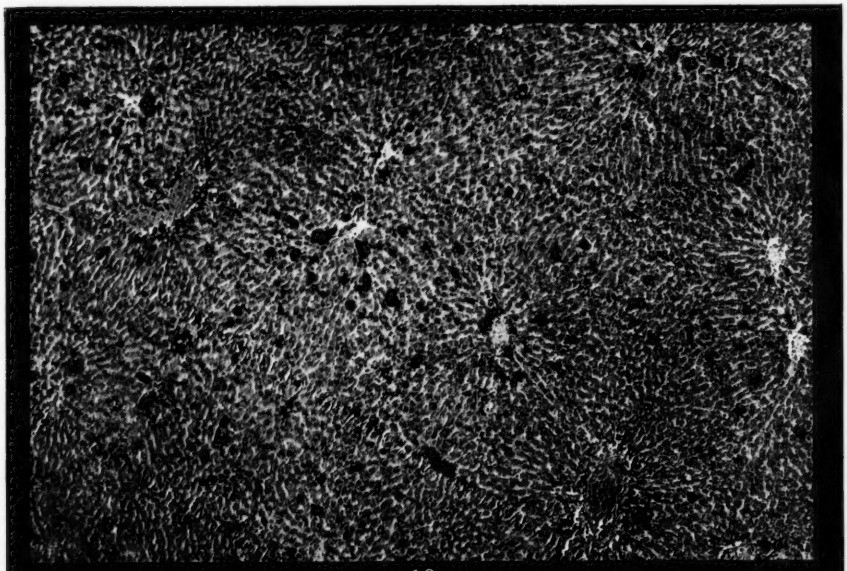
DESCRIPTION OF PLATES.

FIG. 10.—Liver of rat which received intravenously 0·4 ml. thrombokinase followed by 16 mg. carbon/100 g. and was killed 1½ hr. later. Note carbon particles in Kuppfer cells especially those located around portal tracts.

FIG. 11.—Liver of rat given 16 mg. carbon/100 g. intravenously and killed 1½ hr. later. The carbon is evenly distributed in the Kuppfer cells throughout.

FIG. 12.—Lung of rat given 0·4 ml. thrombokinase followed by 16 mg. carbon/100 g., and killed 1½ hr. later. There is a definite quantity of carbon in the capillaries which in places appear lined with carbon.

FIG. 13.—Lung of rat given 0·4 ml. thromboplastin followed by 16 mg. carbon/100 g., and killed 48 hr. later. There is much less carbon than in Fig. 12. Particles of carbon are seen inside intra-alveolar cells; and in a large vessel large cells are filled with India ink.



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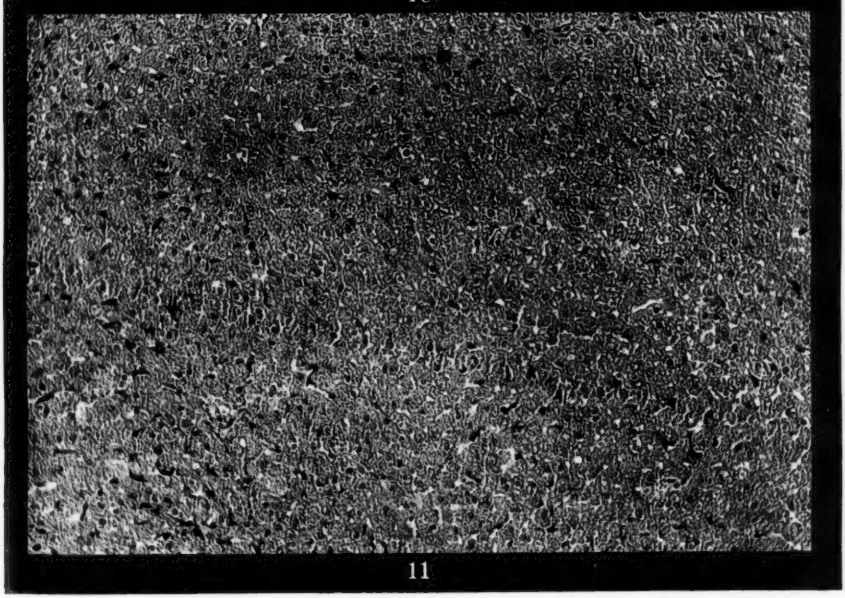

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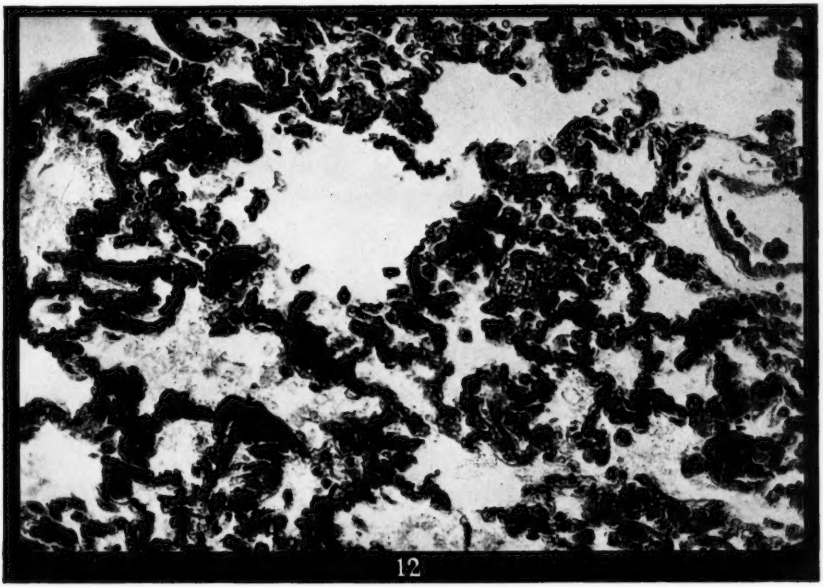

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It should be stressed also that in the animals treated with the coagulant, the capillaries of the lungs were uniformly covered with a layer of carbon (Fig. 12). In the kidneys of these animals, particles of carbon were found in the glomeruli, which seemed to be etched by the carbon.

If the animals are killed 48 hr. later, the histological aspect of the organs is different. A considerable amount of the ink has disappeared from the lungs, but the particles of carbon remaining are inside the intra-alveolar cells. One can observe inside the larger pulmonary vessels some large cells filled with India

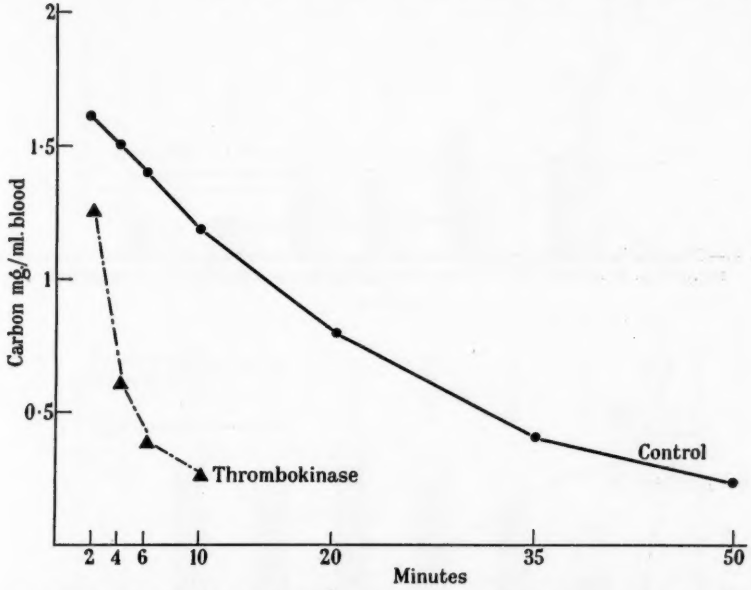

FIG. 3.—Effect of thromboplastin (thrombokinase) on rate of blood clearance of carbon particles in mice following intravenous injection of 24 mg./100 g. (commercial ink Pelikan).

ink (Fig. 13). The liver and spleen, however, showed a greater accumulation of carbon than was found in the animals killed earlier.

In order to verify quantitatively the impression gathered from histological study we analysed the various organs for carbon at regular intervals during the hours following the administration of carbon and thromboplastin (Fig. 4). The values obtained confirm that the carbon particles migrate rapidly from the lungs to the liver and spleen.

Substances which Prevent Blood Coagulation.

Action of heparin in rats.—The rates of clearance of carbon from the blood in heparinized rats are presented in Fig. 5 for various doses of carbon, together with those found in controls. The data represent the mean of the results obtained in 5 rats for each dose.

It can be seen that when 8 mg. carbon/100 g. are injected heparin does not

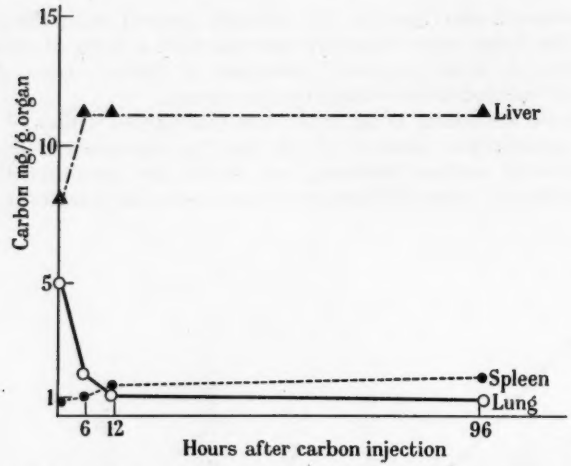

FIG. 4.—Changes in distribution of carbon in rat organs following intravenous injection of 16 mg./100 g. in animals which received an intravenous injection of thromboplastin.

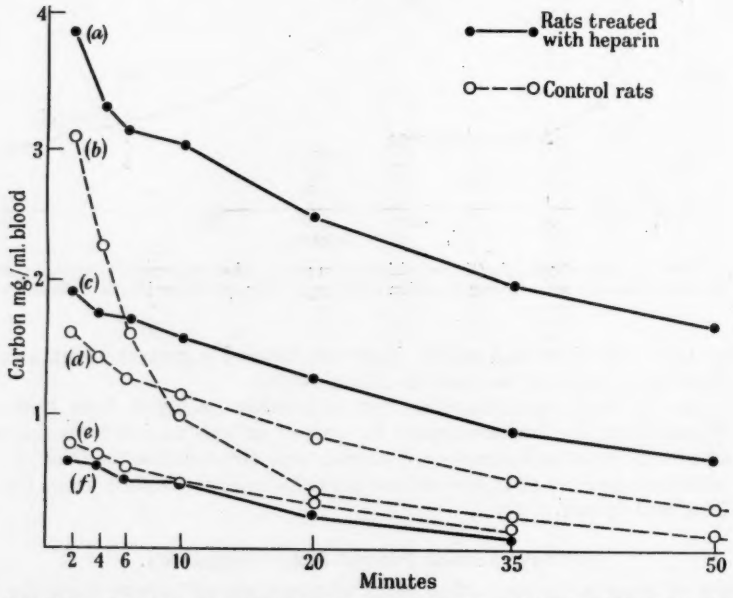

FIG. 5.—Effect of an intravenous injection of 5 mg. heparin on the rate of clearance of carbon from the blood of rats for various doses of carbon (commercial ink Pelikan).

Dosage of carbon : (*a*) & (*b*) 32 mg./100 g.
(*c*) & (*d*) 16 mg./100 g.
(*e*) & (*f*) 8 mg./100 g.

affect the rate of disappearance of carbon particles from the blood. When 16 mg./100 g. are injected into rats previously treated with heparin, the rate of clearance of carbon from the blood is distinctly slowed. This effect is well outside the standard deviation for this dose of carbon (Halpern *et al.*, 1951). The animals treated with heparin which received 32 mg. carbon/100 g. behaved in a way quite different from the control animals. The clearance of the carbon

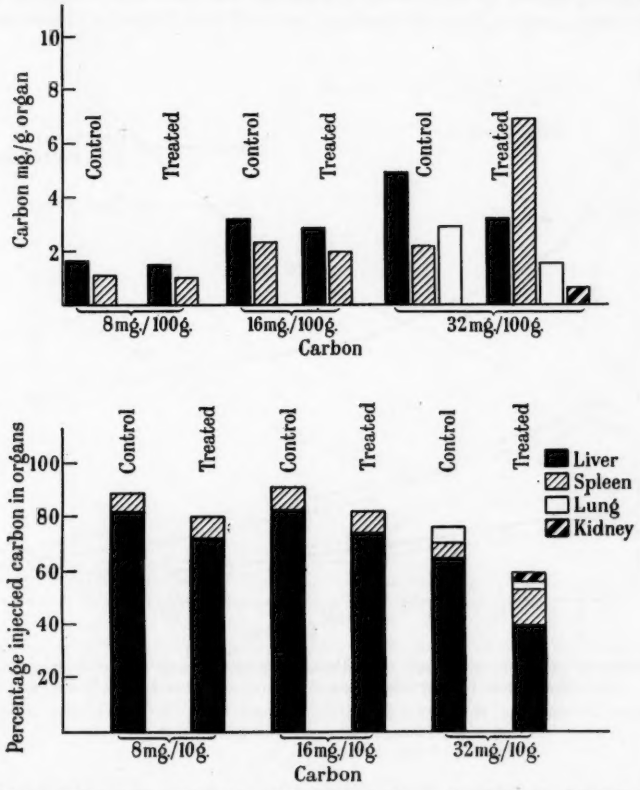

FIG. 6.—Effect of intravenous injection of 5 mg. heparin on distribution of carbon to various organs when different doses of carbon are injected intravenously in rats (commercial ink Pelikan).

particles from the blood was very slow and progressive, and the amount present after 50 min. was still 1·7 mg./ml. as compared with 0·1 mg./ml. in animals not given heparin. This difference is well outside the standard deviation for this dose of carbon.

A study of the distribution of the carbon in the organs is presented (Fig. 6). The analyses were carried out in 6 rats (2 rats per dose). The values are given as the means of the results obtained and compared with those found in control animals receiving the same dose of ink. There is no significant difference in the

distribution of the carbon in the various organs (liver, spleen, lung) between the treated and the control animals when 8 mg. or 16 mg./100 g. are injected. There is a small difference in the total carbon recovered, the amount being less in the treated animals. This can be attributed to the loss of carbon due to the repeated small haemorrhages in the heparinized animals when samples of blood are taken.

There are, however, important differences between the treated and the control rats in the group given 32 mg./100 g. Besides the fact that the total amount of carbon recovered is again lower in animals treated with heparin, there are consider-

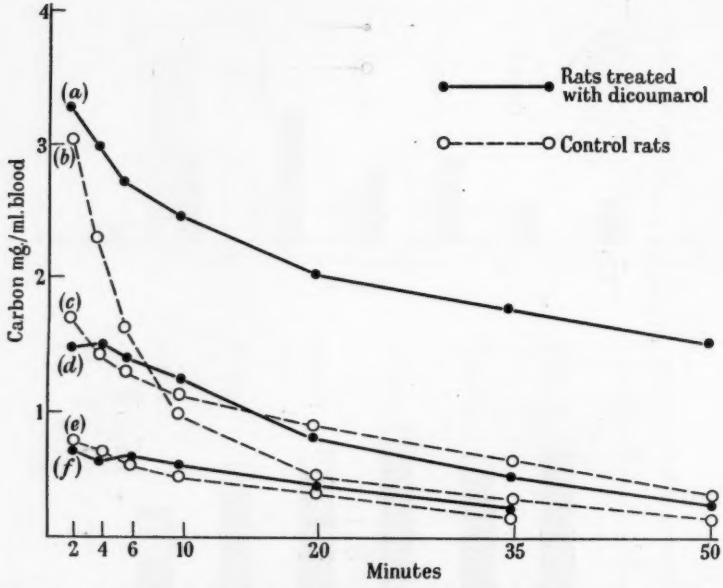

FIG. 7.—Effect of previous treatment with dicoumarol on the rate of blood clearance of carbon particles in rats for various doses of carbon (commercial ink Pelikan).

Dosage of carbon: (*a*) & (*b*) 32 mg./100 g.
(*c*) & (*d*) 16 mg./100 g.
(*e*) & (*f*) 8 mg./100 g.

able changes in the distribution of carbon in the various organs. The lungs of the treated animals contain only about 50 per cent of the amount of carbon found in the lungs of the control rats. On the other hand, the spleen of heparinized animals takes up three times more carbon than that of controls. The liver in animals treated with heparin contains on the average 30 per cent more carbon than in the controls.

Effect of dicoumarol in rats.—The results obtained with dicoumarol resemble those obtained with heparin except that its action is not as effective. The rate of clearance of carbon from the blood is not affected when either 8 mg. or 16 mg. carbon/100 g. are injected. However, when 32 mg./100 g. are injected there is a considerable slowing of the rate of clearance of carbon from the blood (Fig. 7).

In the heparinized animal this effect can be already observed with the dose of 16 mg./100 g. The distribution of carbon in the various organs of the animals treated with dicoumarol is identical with that of the animals treated with heparin for the same doses of ink.

Effect of heparin in rabbits.—When 8 mg. carbon/100 g. are injected, treatment with heparin does not modify the rate of clearance of carbon from the blood in the rabbit (Fig. 8). However, when 16 mg./100 g. are injected the rate of clear-

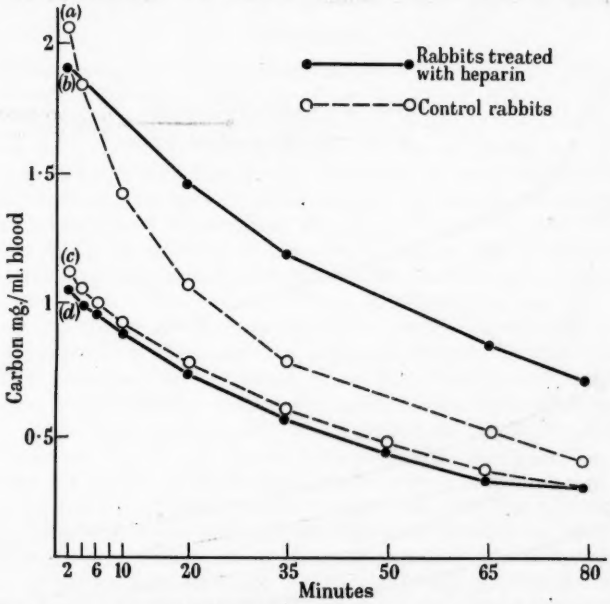

FIG. 8.—Effect of 25 mg. heparin injected intravenously on rate of blood clearance of carbon particles in rabbits when 8 mg. or 16 mg. carbon/100 g. (commercial ink Pelikan) are administered intravenously.

Dosage of carbon: (*a*) & (*b*) 16 mg./100 g.
(*c*) & (*d*) 8 mg./100 g.

ance from the blood is changed appreciably by the previous administration of 50 mg. of heparin. This dose of ink in the rabbit apparently causes a significant microcoagulation of fibrin.

Injection of a Suspension of Carbon Particles without Shellac.

The data presented in Fig. 9 show that when 32 mg. carbon/100 g. of a carbon suspension without shellac is injected, the rate of clearance from the blood differs greatly from that seen when commercial ink containing shellac is administered. When 8 mg./100 g. are injected there is no significant difference between the two curves of clearance. When 16 mg./100 g. are injected the rate of clearance of carbon from the blood in the case of the ink without shellac is somewhat slower

than that seen with the shellac-containing ink, but is identical with the rate of clearance observed in the animals heparinized before receiving the shellac-containing ink (Fig. 5). When 32 mg./100 g. without shellac are injected the rate of clearance is markedly slower than that found in the animals receiving carbon with shellac, and somewhat slower than that of animals treated also with heparin, as heparin is not able to prevent all blood coagulation for this high dose of shellac.

These results show that it is the shellac which is responsible for the phenomenon of blood-clotting *in vivo*, which causes in turn the flocculation of the carbon particles.

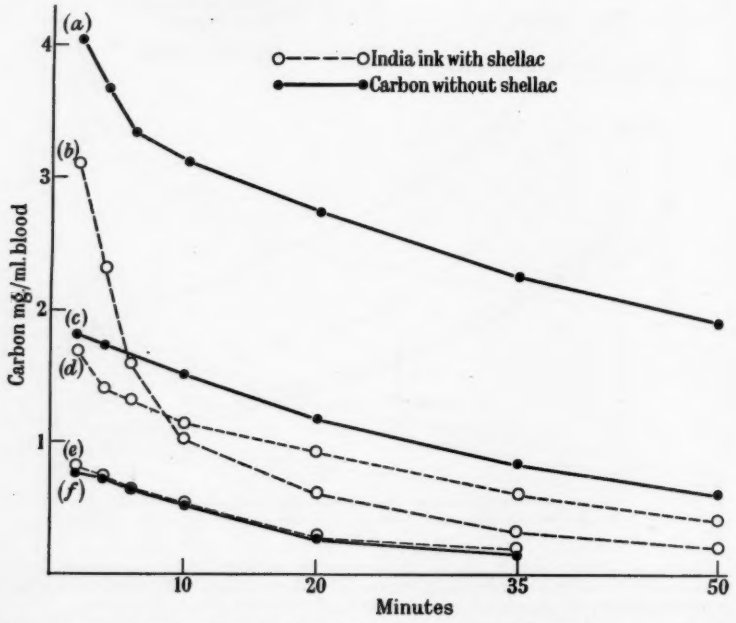

FIG. 9.—Clearance of carbon from the blood in rats following intravenous injection of equivalent doses of carbon as (i) a suspension of carbon without shellac, and (ii) commercial India ink containing shellac.

Dosage of carbon: (*a*) & (*b*) 32 mg./100 g.
(*c*) & (*d*) 16 mg./100 g.
(*e*) & (*f*) 8 mg./100 g.

It should be noted also that the animals given carbon suspension without shellac, even in large doses (up to 48 mg./100 g.) behaved normally and survived indefinitely. This showed that the carbon particles themselves were not toxic.

The amount of carbon recovered from the organs for the various doses of ink showed that there were no differences between animals given ink with shellac and those given the ink without shellac for the doses of 8 and 16 mg. of carbon/100 g. However, when 32 mg. of carbon without shellac are injected only a negligible amount of carbon was found in the lungs and none in the kidneys; almost

all the injected carbon was found in the liver and spleen. When carbon without shellac was administered the carbon was found evenly distributed in the Kupffer cells in the liver and the reticular cells of the spleen for all the doses studied.

A study to determine whether the shellac found in the commercial India ink had coagulative properties showed that these were absent The blood-clotting effect determined *in vivo* by the ink with shellac should therefore be attributed to a release of thromboplastin from the tissues by a mechanism as yet undetermined.

DISCUSSION.

These experiments show that the substances which interfere with the coagulation of blood modify the rate of clearance from the blood of carbon injected intravenously. In order to make this discussion clear it may be recalled that in normal animals (rats, mice, rabbits) there is a critical dose of India ink differing with animal species, below which the rate of clearance of particles from the blood is a regular exponential function of time, and above which the rate of clearance of carbon is considerably accelerated and irregular. With the lower doses of ink containing shellac practically all the carbon is fixed in the reticulo-endothelial cells of the liver and spleen, and with the higher doses of ink a great quantity is retained in the lungs. This radical change is related, as has been shown previously, to an imbalance of the factors which control blood coagulation *in vivo*, and is probably due to a liberation of a great quantity of thromboplastin.

The results presented in this study show that in rats and mice, substances which promote blood clotting render the rate of clearance of carbon from the blood much more rapid and modify the distribution of carbon in the various organs (in the same way as high doses of ink containing shellac in the normal rat).

The substances which promote blood clotting were used in the rat with a dose of 16 mg. of carbon/100 g. which, as we have shown, does not change appreciably the number of blood platelets or the level of plasma fibrinogen. This same effect of the substances promoting blood clotting can be demonstrated using even smaller doses of carbon such as 8 mg./100 g., but in this case the amount of blood-clotting agent to be injected seems to vary inversely with the square of the dose of carbon. When large doses of ink containing shellac are injected or when blood coagulants are injected with small doses of carbon, one can observe the accumulation of carbon in the lungs and kidneys, which normally do not show this phenomenon. The retention of carbon in the pulmonary vessels determined by the coagulation of fibrin is only transient, and slow displacement follows from the lungs to the liver and spleen (Fig. 12). This indicates that the mechanism of the fixation of carbon in the lungs is very different from the true phagocytosis by reticulo-endothelial cells.

Since the substances which promote the coagulation of blood accelerate the clearance of carbon of the blood and modify its distribution in the various organs, one might expect that anticoagulants would effect these reactions in the other direction. We have seen, however that the curve of clearance of carbon for the dose of 8 mg. carbon/100 g. is not changed by anticoagulants, and is identical with that found when the same dose of carbon without shellac is injected. This confirms the fact that in rats and rabbits the dose of 8 mg. carbon/100 g. of commercial India ink does not cause the coagulation of the blood *in vivo*. Moreover it proves also that the phagocytic activity of reticulo-endothelial cells of the liver

and spleen is not affected by a lack of coagulability of the blood, and is not dependent upon the coagulation of fibrin.

When 16 mg. of carbon/100 g. of ink containing shellac are injected into rats treated with heparin, the rate of clearance of carbon from the blood is slower than in control animals although the distribution of carbon in the various organs is not changed. This confirms that this dose of ink causes only a small amount of coagulation of fibrin *in vivo* which slightly modifies the size of the carbon particles, but is not sufficient to cause their retention by the lungs.

Animals treated with dicoumarol with a prothrombin level below 5 per cent of normal do not behave differently from normal animals when a dose of ink of 16 mg. carbon/100 g. is injected. This difference between the effect of heparin and dicoumarol for this dose of carbon can be explained by the difference in the mechanism of these anticoagulants. Heparin is a substance which is believed to inhibit blood coagulation at its various intermediate stages (Eagle, 1937). Dicoumarol is an anticoagulant which acts by blocking the formation of prothrombin. The level of prothrombin remaining in rats treated with dicoumarol may be sufficient to cause the small amount of coagulation of fibrin which is produced in rats by the dose of 16 mg. carbon/100 g.

It must be stressed, however, that both heparin and dicoumarol have a very important effect upon the clearance of carbon from the blood in rats given 32 mg./100 g. in ink containing shellac. In spite of its very powerful anticoagulant action heparin is not able to inhibit the coagulating effect of a high dose of carbon with shellac completely, since a small quantity of carbon is still present in the lungs of these animals. This shows how large a release of thromboplastin is determined by the shellac present in the ink.

The phagocytic power of the spleen appears considerably increased in the heparinized animal or in the animal given carbon without shellac. This greater activity of the spleen can be attributed again to the fact that in these animals the particles of carbon remain in the blood much longer, and not to a greater activity of the splenic cells themselves.

These findings indicate that the mechanism by which carbon particles are taken by the lungs is determined by a coagulation of fibrin *in vivo*. Moreover, it must be emphasized that it is the shellac injected with the carbon particles which is responsible for the liberation of thromboplastin from the tissues by an unknown mechanism.

The following previous work should be reconsidered in the light of these findings. Jancso using colloidal gold reported in 1931 that in mice, treatment with heparin decreased the granulopectic activity of the reticulo-endothelial system. The results of our experiments do not confirm these observations. Recently Sheppard, Gilbert and Hahn (1951) working also with colloidal gold in dogs reported that heparin was unable to influence granulopectic activity.

Timiras and Selye (1949) and Timiras (1953) observed the fixation of carbon particles in the lungs of rats injected with commercial India ink under the effect of "stress," and interpreted it as a true granulopectic activity under the influence of the "alarm reaction." It is quite probable that they have observed a deposition of carbon in the lungs of their animals determined by changes in the equilibrium of the coagulation of blood under the influence of various forms of stress. Moreover the doses of ink used by these investigators alone caused a flocculation of carbon particles *in vivo* and their retention by the lungs.

We suggest that the mechanism demonstrated in this study is responsible for the findings of these authors.

Our experiments also afford a possible explanation for the observations of Sanders, Florey and Wells (1951) and Markham and Florey (1951). These authors reported that when they injected a dilution of commercial India ink into rabbits, they saw the ink disappear very rapidly from the blood. The particles of carbon coalesced to form larger aggregates, and concomitantly they observed an important fixation of carbon by the lungs. It should be mentioned that these authors injected the ink without a protective colloid such as gelatin, and we have observed that coagulation of blood *in vivo* with accumulation of the ink in the lungs will occur with much smaller doses of ink when gelatin is not used to stabilize the suspension.

The rate of blood clearance and distribution of carbon in the organs are not affected either by anticoagulants or by the presence of shellac when a dosage of 8 mg./100 g. is employed. This indicates that when using commercial ink to study the phagocytic activity of the liver and spleen this dosage must not be exceeded if errors caused by coagulation of fibrin *in vivo* are to be avoided. If higher dosage is required it is preferable to use a suspension of carbon without shellac such as the one we have described in this study.

SUMMARY.

Thrombin or thromboplastin, when administered intravenously, increase the rate of blood clearance of carbon particles and modify the distribution of carbon in the organs.

The action of substances promoting blood coagulation is therefore very similar to that of high doses of commercial India ink (carbon with shellac) alone. This confirms the hypothesis that high doses of these inks cause a microcoagulation of fibrin *in vivo*.

The action of anticoagulant differs according to the dose of India ink injected. Small doses of carbon modify neither the rate of clearance of carbon particles of the blood nor their distribution in the various organs. When high doses of ink are injected a considerable decrease in the rate of clearance of carbon of the blood is observed. The amount of carbon in the lungs of animals treated with the anticoagulants is smaller and that in the spleen greater than in controls.

The coagulation of fibrin *in vivo* caused by higher doses of commercial India ink as well as its toxicity are attributable to its shellac content.

In a study of the activity of the reticulo-endothelial system only specially prepared suspensions of carbon without shellac should be used. If commercial India ink containing shellac is injected, care should be taken to administer doses which do not cause a release of thromboplastin *in vivo*.

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QUANTITATIVE STUDY OF THE GRANULOPECTIC ACTIVITY OF THE RETICULO-ENDOTHELIAL SYSTEM. II: A STUDY OF THE KINETICS OF THE GRANULOPECTIC ACTIVITY OF THE R.E.S. IN RELATION TO THE DOSE OF CARBON INJECTED. RELATIONSHIP BETWEEN THE WEIGHT OF THE ORGANS AND THEIR ACTIVITY.

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In previous studies (Halpern, Biozzi and Mené, 1950; Biozzi, Benacerraf, Mené and Halpern, 1951; Halpern, Biozzi, Mené and Benacerraf, 1951*a*), it was shown that most of the results obtained with commercial India ink by authors investigating the activity of the R.E.S. (Timiras, 1953; Markham and Florey, 1951) were vitiated by the toxic reactions caused by the shellac present in these India ink preparations, in spite of the fact that stable homogeneous and well-dispersed suspensions of carbon were used. It was established that in the rat when doses of India ink above 16 mg. carbon/100 g. were administered, the shellac present in the ink* determined a liberation of thromboplastin *in vivo* which initiated the coagulation of fibrin in the circulation. This phenomenon caused flocculation of the carbon particles, which were then arrested by the lung capillaries.

Doses of ink above 16 mg. carbon/100 g. also caused the death of the animals within a few hours. In a study of the granulopectic activity of the R.E.S., results obtained with commercial India ink containing shellac can only be interpreted when these considerations are taken into account.

When doses of commercial India ink below 16 mg. carbon/100 g. were administered intravenously (Halpern *et al.*, 1951*a*) the rate of clearance of particles from the blood followed a simple mathematical law where the concentration in the blood is an exponential function of the time. Almost all the carbon injected is then found in the liver and spleen. It was observed also (Halpern *et al.*, 1951*b*) that the previous injection of a small dose of India ink (8 mg. carbon/100 g.) slowed the rate of clearance of the carbon particles when subsequent injections of the same dose were given. These results showed that India ink with shellac determined a relative saturation of the granulopectic function of the R.E.S. for particles of about 250 Å. However, the studies with this carbon suspension containing shellac could not be carried on any further due to the untoward effect of the shellac.

A carbon suspension in fish glue free from shellac was specially prepared for us by Gunther Wagner, Hanover. This suspension of carbon contained particles homogeneous in size (as in commercial ink Pelikan about 250 Å) and only fish glue

* Commercial India ink, "Pelikan," Gunther Wagner, Hanover.

as a preservative agent. Experiments showed that the fish glue present in the ink was not toxic and affected neither the granulopectic function of the R.E.S. nor the blood-clotting mechanism. Moreover this suspension of carbon was very stable in the serum and non-toxic for all the doses used.

This allowed us to undertake a more complete study of the granulopectic function of the cells of the reticulo-endothelial system in contact with the blood. We are reporting here the data obtained in the rat when various doses of this special suspension of carbon are injected intravenously. The rate of clearance of carbon from the blood and the distribution of carbon to the various organs were studied. Mathematical formulae expressing the granulopectic function of the cells for these particles are proposed. These mathematical expressions established the relationship found in the rat between the speed of the granulopectic activity and the dose of carbon administered and the weight of the organs (liver and spleen) principally responsible for the granulopectic activity for particles of this size. On the basis of these results an explanation of the mechanism which governs the granulopectic activity of the cells of the R.E.S. is offered.

EXPERIMENTAL.

Effect of the dose of carbon injected on the rate of clearance of carbon from the blood and its distribution in the organs.

MATERIALS AND METHODS.

A suspension of carbon (C 11/1431 a) specially prepared as described was used throughout the experiments. This preparation contained about 100 mg. carbon/ml. suspended in a solution of fish glue with a small amount of phenol as preservative. The carbon suspension was centrifuged at 5000 r.p.m. for 15 min. in order to get rid of the small number of large particles of carbon present in the original preparation. The supernatant was analysed for carbon by weight after the carbon had been precipitated by concentrated NaOH and the proteins hydrolysed by boiling. It contained generally about 90 mg. carbon/ml. This suspension of carbon was then diluted with a solution in double distilled water of gelatin neutralized with ammonia. Dilutions of this ink containing 8 mg. carbon and 0·5 per cent gelatin, 16 mg. carbon and 1 per cent gelatin, and 32 mg. carbon and 2 per cent gelatin were prepared. The suspensions were kept at about 40° before injection to avoid solidification of the gelatin.

White male rats weighing 80–160 g. were given doses of 4, 8, 16, 32 and 48 mg. carbon/100 g. intravenously. To administer the dose of 4 mg. carbon/100 g. a dilution of ink containing 8 mg./ml. was used ; to inject the dose of 8 mg. and 16 mg. carbon/100 g. a dilution of ink containing 16 mg. carbon/ml. was injected, and for higher doses a dilution of ink containing 32 mg. carbon/ml. All injections were made into the dorsal vein of the penis in the unanaesthetized animal. The rate of clearance of the carbon particles from the blood was investigated as described previously (Halpern *et al.*, 1951*a*, *b*). Blood samples were obtained at regular intervals by puncturing the retro-orbital venous plexus (Halpern and Pacaud, 1951) with a fine capillary glass pipette previously washed with heparin and calibrated to 0·025 ml. This quantity of blood was then lysed in 2 ml. of 0·1 per cent Na_2CO_3 and the amount of carbon in the blood determined electrophotometrically in red light. This procedure was repeated as many times as necessary as the animals are not affected by the bleedings. After the carbon had completely disappeared from the blood all the animals were sacrificed and the weights of the organs (liver and spleen) recorded.

In a group of animals having received the various doses of carbon studied, the livers, spleens, lungs and kidneys were analysed for carbon as described previously (Halpern *et al.*, 1951*a*, *b*). The whole organs were hydrolysed in boiling NaOH under a layer of ethyl alcohol to absorb the fatty substances and after washing and drying the recovered carbon was weighed. In another group of animals given the same doses studied a histological study was performed.

RESULTS.

The results presented in Fig. 1 show that for all doses of carbon injected, 4 mg.–48 mg./100 g., the clearance of carbon from the blood is regular and progressive. If the logarithm of the concentration of carbon is plotted against the time as in Fig. 2, the curves of clearance of carbon with respect to time are straight lines except for the highest doses of carbon, 32 mg. and 48 mg./100 g., and only

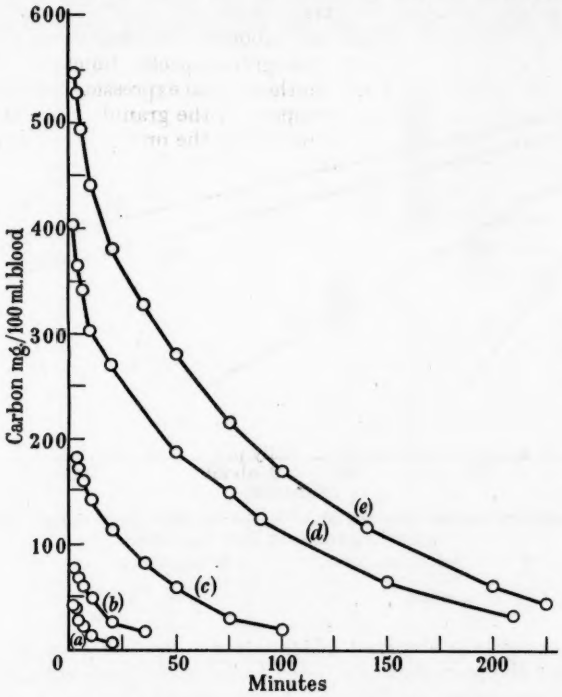

FIG. 1.—Clearances of carbon from the blood in the rat after injection of single doses.

(*a*) 4 mg./100 g. (*d*) 32 mg./100 g.
(*b*) 8 „ „ (*e*) 48 „ „
(*c*) 16 „ „

for the first few minutes. These abnormal values could be attributed to a small initial flocculation of carbon with these high doses. It can generally be said, however, that when a suspension of carbon is injected the concentration of carbon is an exponential function of the time, and can be expressed by equation 1—

$$\frac{\log Co - \log C}{T} = K \quad \text{or} \quad C = Co\ 10^{-KT},$$

where C is the concentration of carbon in the blood at the time T and Co the blood concentration of carbon just after the injection and before the particles

are absorbed by the R.E.S. K is a constant which characterizes the rate of clearance of carbon from the blood and which we shall call the Granulopectic Index. We can see in Fig. 2 that when the log. of the concentration of carbon is plotted against time, the straight lines obtained for the various doses of carbon injected are not parallel and the higher the dose of carbon the more do we tend towards a horizontal line, which means that the granulopectic index of equation 1 tends to be smaller.

In Fig. 4 we have plotted in ordinates the mean values of K, granulopectic index, for each dose of carbon and the corresponding doses of carbon injected

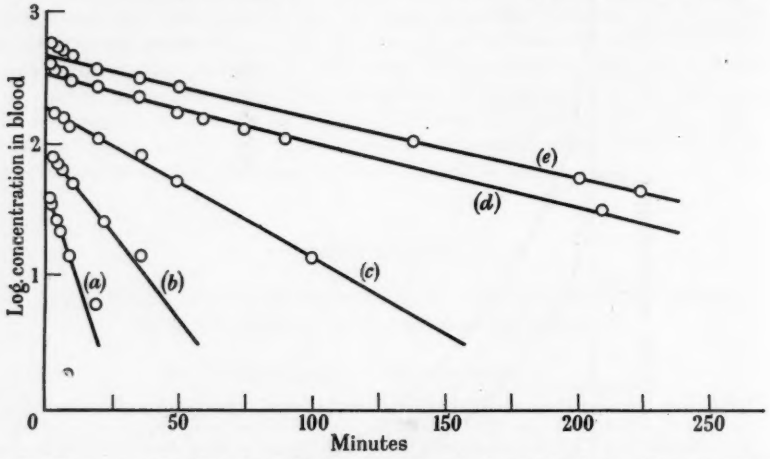

FIG. 2.—Clearances of carbon from the blood in the rat after single doses. Concentrations of carbon expressed as their logarithms.

(*a*) 4 mg./100 g. (*d*) 32 mg./100 g.
(*b*) 8 „ „ (*e*) 48 „ „
(*c*) 16 „ „

in abscissae. A curve is obtained which is very close to an equilateral hyperbola. This means that the granulopectic index is inversely proportional to the dose of carbon injected (equation 2).

$$D \times K = 0{\cdot}208,$$

where K is the constant from equation 1 and D the dose of carbon injected. The dose of carbon injected is the product of the initial blood concentration × the blood volume. If we take the blood volume of the rat to be 8 ml./100 g., which is close to what has been found with our method by extrapolating our carbon clearance curves in Fig. 2 to the time O, equation 2 becomes $C_O \times K = 0{\cdot}026$.

Thus we can replace K in equation 1 by its value from equation 2 :

$$K = \frac{0{\cdot}026}{C_O};$$

we obtain equation 3

$$C = C_O 10^{-\frac{0{\cdot}026}{C_O} T}.$$

In order to investigate the factors which regulate the speed at which the cells of the R.E.S. absorb the particles we should obtain the derivative of equation 3 with respect to T. This gives equation 4,

$$-\frac{d\,c}{d\,t} = \frac{0{\cdot}026 \times 2{\cdot}3C}{C_o} = 0{\cdot}06\,\frac{C}{C_o},$$

2·3 being the factor of conversion of natural into decimal logarithms.

It appears therefore from equation 4 that the speed at which the carbon is eliminated from the blood and stored in the organs (liver and spleen) is directly

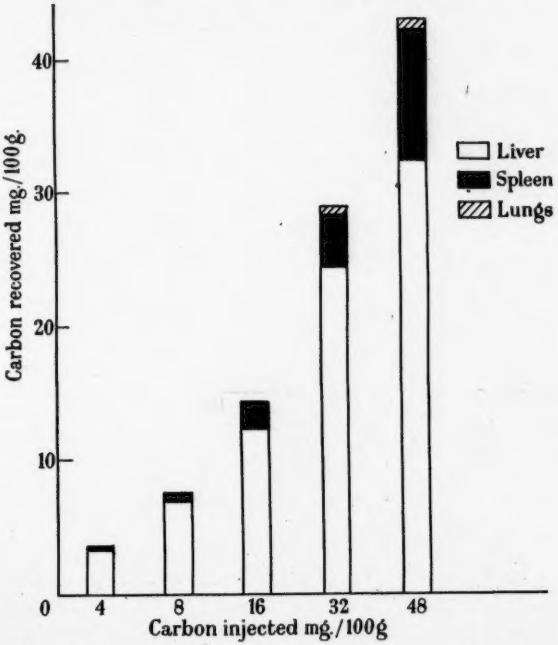

FIG. 3.—Distribution of carbon among the various organs after injection of doses from 4 mg. to 48 mg./100 g.

proportional to its concentration in the blood and inversely proportional to the initial concentration of carbon.

However, equation 4 can be transformed further to reveal more clearly the saturating effect of the carbon particles on the activity of the cells once they are phagocyted. This effect, of which there is ample proof, can be seen in Fig. 1; if the speeds of clearance of carbon for the various doses of carbon injected are compared for the times at which the blood concentrations are identical, it can easily be seen that the rate of clearance of carbon is highest in animals having absorbed least carbon.

Equation 4 can be transformed in the following way: at all times the concentration of carbon in the blood C is equal to the initial concentration Co

less the amount phagocyted by the R.E.S. which we shall call P. Therefore, $C = Co - P$. If we replace C for this value in equation 4 we obtain equation 5,

$$-\frac{d\,C}{d\,t} = 0{\cdot}06\left(\frac{Co-P}{Co}\right) = 0{\cdot}06\left(1-\frac{P}{Co}\right),$$

which is true for all the doses of carbon and expresses the granulopectic activity of the R.E.S. of the liver and spleen for our carbon particles. When P tends to

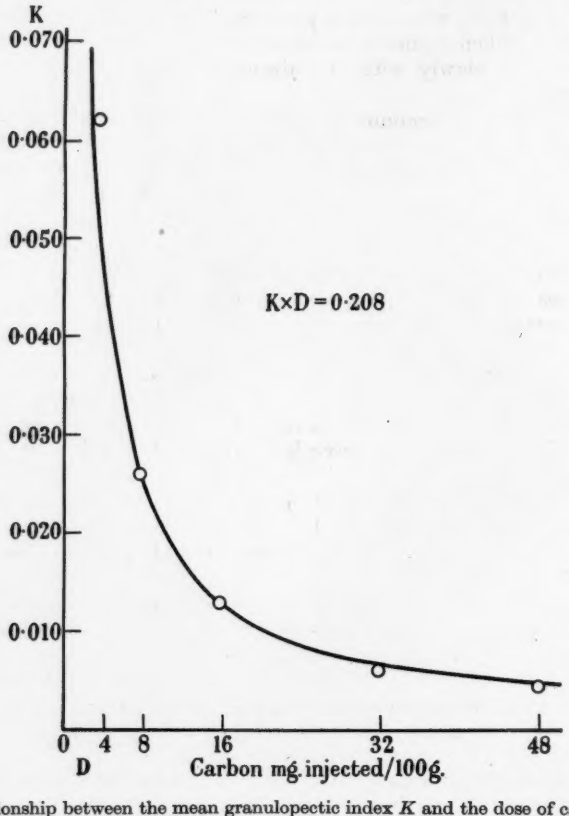

FIG. 4.—Relationship between the mean granulopectic index K and the dose of carbon injected in the rat.

be very small (that is at the beginning of the experiment), $\frac{P}{Co}$ tends to be very small and $\frac{d\,C}{d\,T}$, which is the rate of clearance of carbon particles, approaches its maximal value 0·06 mg./min./ml. As P increases, that is as a greater ratio of the injected carbon is taken by the cells, $\frac{P}{Co}$ tends towards 1 and $\frac{d\,C}{d\,T}$ approaches O.

As equation 5 is valid for all the doses studied from 4 mg. to 48 mg. of carbon/100 g., it follows that the initial speed of granulopectic activity of the cells of the R.E.S. is the same for all the doses and initial blood concentrations used, going from 0·5 mg./ml. to 12 mg./ml. It is its maximal possible speed of phagocytosis for these particles. It can be deduced from equation 5 that the cells of the R.E.S. are saturated by the carbon which they take, as the speed of clearance decreases regularly with the amount of carbon which goes inside the cells. It must be stressed, however, that this saturating effect of the absorbed carbon in the R.E.S. or fatigue of the R.E.S., whichever is preferred, is a relative phenomenon and not an absolute one. When higher doses of carbon are injected the rate of phagocytosis decreases more slowly with the absorbed carbon than when lower doses are administered.

To illustrate this, the amount of carbon/ml. phagocyted by the cells of the R.E.S. is plotted against the time for all the doses of carbon injected. (In order to avoid confusion from the abnormal values found in the first few minutes for the doses of 32 and 48 mg./100 g., these were replaced by the corresponding values extrapolated from the respective curves in Fig. 2.)

It can be seen in Fig. 5 that the initial rate of phagocytosis is the same for all doses. However, granulopectic activity decreases much faster for lower than for higher doses of carbon. What really determines the rate of phagocytosis of the carbon particles by the cells is not the actual amount of carbon phagocyted, but rather its ratio to the total amount injected. It follows from this that for all the doses of carbon the same speed of phagocytosis is observed when, for instance, half of the carbon injected has been taken by the cells. This means that the R.E.S. cells clear the carbon from the blood at the same speed for the dose of 4 mg./100 g. when 2 mg. have been absorbed and for the dose of 48 mg./100 g. when 24 mg./100 g. have been phagocyted.

Therefore it seems that the granulopectic activity of the R.E.S. cells of the liver and spleen for the carbon particles is stimulated by the carbon injected and inhibited by that which they have absorbed, according to the law expressed in equation 5.

The saturating effect of the carbon phagocyted explains the exponential nature of the curve of clearance. If the carbon particles did not saturate the cells of the R.E.S. as they are taken, the curves of clearance with respect to time would always be the same straight line ; this line has been drawn in Fig. 5 for comparison.

Further proof of the stimulating influence of the circulating carbon particles and of the saturating effect of the carbon phagocyted on the cells of the R.E.S. will be brought later. It should be stressed at this point, however, that all the values from which the equations 1 to 5 are derived have been obtained from the mean values of all the observations on a very large number of rats in order to offset the influence of the individual variations which are very large. These individual variations compensate for each other very adequately when a large number of animals are used. They are related principally to the weight of the organs directly involved.

The results presented in Fig. 2 show the quantity of carbon recovered in the various organs, liver, spleen, lungs and kidney for the various doses of carbon injected. About 90 per cent of the injected carbon is generally recovered, principally in the liver and spleen. For the doses of 4 mg., 8 mg., 16 mg./100 g. the liver is responsible for the greater part of the granulopectic activity, the spleen

taking an amount of carbon in relation to its weight. For the doses of 32 and 48 mg./100 g. there is a very small amount of carbon in the lungs (less than 1 mg.) which may be attributed to slight flocculation of the particles for these high doses. Although the amount found in the liver increases for these high doses, the amount found in the spleen increases more considerably when one considers that the average weight of the spleen is about 0·600 g./100 g. while that of the liver is 4·5 g./100 g. of rat.

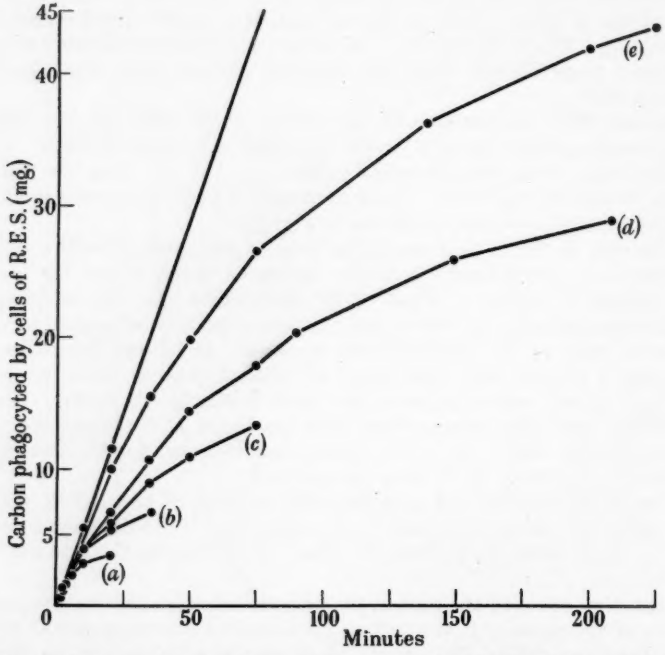

FIG. 5.—Speed of phagocytosis of carbon particles by the cells of the R.E.S. for various doses of carbon. The straight line represents the speed of phagocytosis of carbon particles in a theoretical rat whose cells would not be saturated by the particles phagocyted.

(*a*) after dose of 4 mg./100 g. (*d*) after dose of 32 mg./100 g.
(*b*) „ „ 8 „ „ (*e*) „ „ 48 „ „
(*c*) „ „ 16 „ „

Effect of Successive Doses of 8 mg. Carbon/100 g. on the Rate of Clearance of Carbon Particles from the Blood.

In order to investigate further the saturating effect of the carbon particles on the granulopectic activity of the R.E. cells, which can be deduced from equation 5

$$-\frac{d\,C}{d\,T} = 0{\cdot}06\left(1 - \frac{P}{Co}\right)$$

we injected intravenously into 10 rats four successive doses of 8 mg. carbon/100 g. Each injection was made as soon as the blood was free from carbon particles. The rate of clearance of carbon from the blood was studied as before. The results are presented in Fig. 6 as the mean for all the values obtained.

The previous injection of one or more doses of 8 mg. carbon/100 g. slows down considerably the rate of clearance of carbon for the following doses. The average concentration found at 35 min. for the first curve of clearance was 0·1 mg./ml. and for the fourth curve of clearance was 0·45 mg./ml. The granulopectic index K calculated from equation 1,

$$C = Co\ 10^{-KT},$$

also changes considerably with successive doses ; it is 0·026 for the first dose, 0·019 for the second, 0·013 for the third and 0·010 for the fourth. The phagocytosis of carbon by the cells of the R.E.S. decreases their granulopectic activity

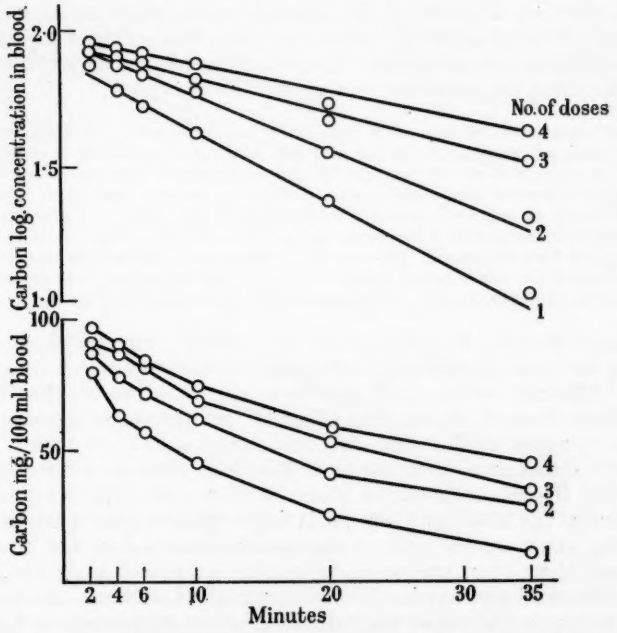

FIG. 6.—Clearances of carbon from the blood in rats when repeated doses of carbon are injected (mean values for 10 rats). All doses = 8 mg./100 g.

when they are challenged again by the same concentration of carbon. This indicates the saturating effect of the phagocyted carbon on the activity of the cells. It should be kept in mind however that the phagocytic activity of the cells is conditioned by the saturating effect of the carbon absorbed, but only in relation to the stimulating effect on these cells of the blood concentration of carbon.

It is interesting to note also that the carbon particles saturate the R.E.S. progressively while they are phagocyted during the clearance of a dose of carbon from the blood and yet the curve of clearance follows an exponential equation. This is another proof that the saturating effect of the particles of carbon on the cells determines the exponential character of the blood-clearance curve.

Effect of a Sudden Increase or Decrease of the Concentration of Carbon in the Blood on the Rate of Clearance from the Blood of a Given Dose of Carbon.

The factors which control the rate of phagocytosis of carbon particles by the cells of the R.E.S. seem to be the concentration of the injected carbon in the blood acting as a stimulating factor and the amount of carbon taken by the cells acting as an inhibiting factor according to equation 5,

$$-\frac{d\,C}{d\,T} = 0{\cdot}06\left(1 - \frac{P}{Co}\right).$$

In order to investigate further these factors and to establish on a firm basis the stimulating effect on the cells of the injected carbon particles as well as the inhibiting effect of the phagocyted carbon particles, two series of experiments were carried out where the concentrations of carbon in the blood were suddenly increased or decreased during the clearance of a given dose of carbon.

In the first experiment a dose of 16 mg./100 g. was injected intravenously in a rat, and enough blood samples were taken in the first few minutes to establish a curve of clearance which could be extrapolated for the rest of the experiment. A very small dose, 2·5 mg., which abruptly increased the blood concentration of carbon, was then injected. Blood samples were taken at regular intervals to establish a new curve of clearance which was compared with what the curve of clearance of carbon would have been if this additional dose of carbon had not been injected. The rate of clearance of carbon was also compared with the initial curve for the same blood concentration. This procedure was repeated. Several such experiments were performed ; a typical result is presented in Fig. 7.

When after 31 min. 2·5 mg. carbon are injected, the speed of clearance of carbon from the blood is increased in relation to what it would have been (dotted line) if no additional carbon had been injected. This shows that the injected carbon particles have a stimulating effect on granulopectic activity. This behaviour can be noted again when the dose is repeated at 59 min. If one compares the rate of clearance of carbon from the blood after the reinjection of carbon at 31 min. with the rate of clearance observed when there was an equal concentration of carbon in the blood at 9 min., it is slower than it was at 9 min. because of the saturating effect on the cells of the carbon absorbed in the interval. This is even more evident when the whole procedure is repeated at 59 min. It follows from these observations that in the same animal when there are equal concentrations of carbon in the blood, the saturating effect on the cells of the difference in amounts of carbon absorbed is evident. When there are equal amounts of carbon in the cells the stimulating effect of an increase of concentration of carbon is again very clear.

In order to *decrease* the concentration of carbon in the blood in the course of the experiment, the following procedure was carried out : A rat was injected with 16 mg. of carbon/100 g. and blood samples taken regularly for the first few min. in order to draw the complete curve of clearance from equation 1. The animal was then rapidly bled from the retro-orbital venous plexus with a calibrated pipette and approximately 2 ml. blood/100 g. were withdrawn. Immediately the same volume of rat serum was injected intravenously to bring back the blood volume to normal. This procedure caused a sudden decrease in the concentration of carbon particles in the blood corresponding to the amount of carbon removed during the bleeding. Blood samples were then taken regularly. The rate of clearance of carbon particles was studied after this procedure and was compared with what it would have been normally. This experiment was carried out over 20 times. It was very delicate,

because care has to be taken to avoid a sustained shock from the procedure which would interfere with the portal circulation and would affect granulopectic activity. Care has to be taken also that the rat serum injected to maintain the blood volume contains only a negligible amount of thrombin, otherwise this could cause coagulation and interfere with the rate of clearance of carbon particles from the blood.

The results of a typical experiment are presented in Fig. 8. If the carbon concentration in the blood is suddenly decreased, the rate of clearance of carbon from the blood diminishes a little; but it should be emphasized that the rate

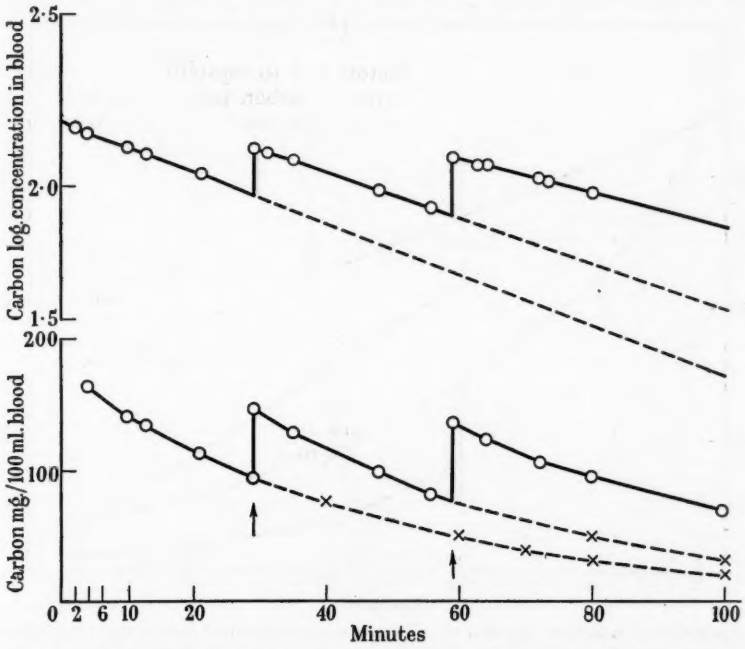

FIG. 7.—Effect of injection of 2·5 mg. carbon on the rate of clearance of carbon from the blood during the clearance of a dose of 16 mg./100 g. Arrows indicate time of injection.

remains much faster than it would have been had the blood concentration of carbon been reduced to the same level by phagocytosis. This behaviour is consistent with the view that the carbon particles once phagocyted by the cells have an inhibiting tendency on their activity, while when they are in the circulation they have a stimulating effect.

Effect of the Weight of the Organs, Liver and Spleen, on the Rate of Clearance of Carbon Particles from the Blood.

The data from the first part of this study were obtained from a large number of animals and are presented as the mean of all the values obtained. There were however, considerable individual variations in granulopectic activity as expressed by the granulopectic index K for a given dose of carbon, especially when

the animals were of different strains and weights. It seemed therefore of interest to investigate whether there was a factor connected with the relative size of the organs endowed with granulopectic activity which allowed some correction to be made.

When a given dose of carbon is injected in the rat, *e.g.*, 8 mg./100 g., the granulopectic index K calculated from equation 1 expresses adequately for the animal studied the granulopectic activity of the cells of the R.E.S. The values of the granulopectic index in different animals can be compared with each other. It

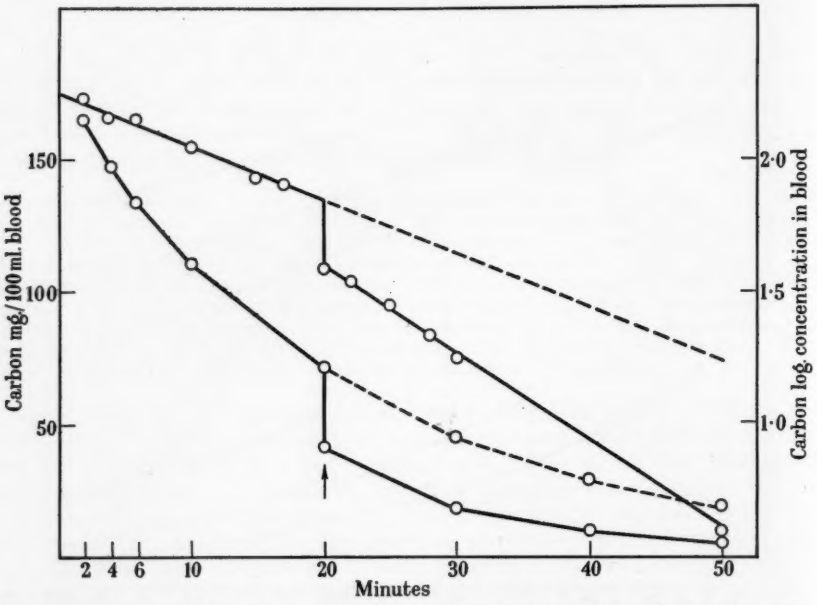

FIG. 8.—Effect of a sudden decrease of the blood concentration of carbon (by bleeding and plasma transfusion), indicated by arrow, on the rate of clearance of carbon from the blood during the clearance of a dose of 16 mg./100 g.

is evident from what has been presented that this is only so when the same dose of carbon is injected into all the animals. The granulopectic indices of two animals receiving different doses of carbon cannot be compared unless they are corrected with the help of equation 3, $Co \times K = 0{\cdot}026$.

In order to study the effect of the weight of the organs principally involved in granulopectic activity, we have studied, for a given dose of carbon, 8 mg./100 g., the variations of the granulopectic index K with respect to the relative weight of the liver and spleen.

The study was performed on 100 rats. The animals received 8 mg./100 g. intravenously; the rate of clearance of carbon from the blood was studied and the granulopectic index K was calculated from equation 1. When all the carbon had disappeared from the blood, the animals were sacrificed and the liver and spleen dissected and weighed immediately in the wet state. This was called $W1s$. As dose of carbon depended on the weight of the animal, the weight of

the organs $W1s$ was compared to the weight of the animal W and the ratio $\frac{W}{W1s}$ calculated for each animal. This value was found to be on the average 20, but it varied from 11 to 30. In Fig. 9 we have plotted in ordinates the granulopectic index K of each animal for the dose of 8 mg. carbon/100 g and in abcissae the ratio $\frac{W}{W1s}$ for the corresponding animal.

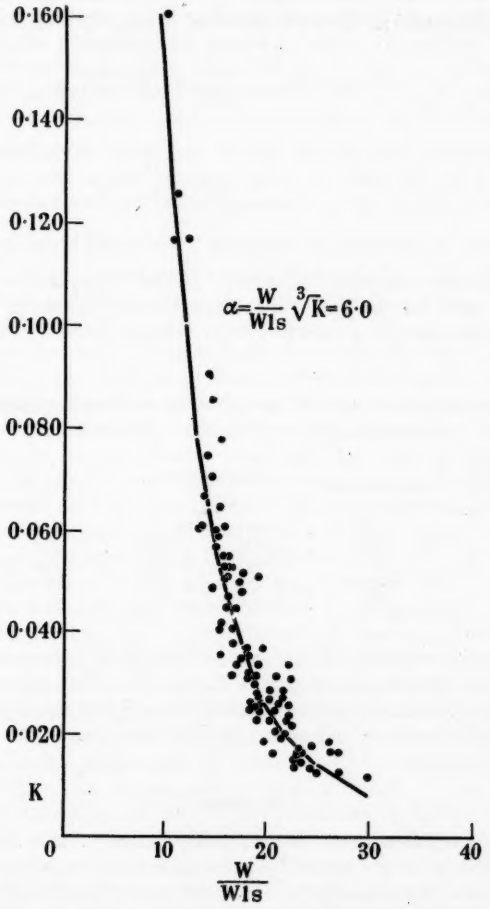

FIG. 9.—Relationship between the relative weight of the liver and spleen $\frac{W}{W1s}$ and the granulopectic index K for the dose of 8 mg. carbon/100 g.

The results presented in Fig. 9 show the spontaneous variations of the granulopectic index for a given dose of carbon. The extreme of the variations were found to be from 0·010 to 0·16, *i.e.*, 16-fold. The relative weights of the liver and spleen can be seen in Fig. 9 to determine directly the value of the granulo-

pectic index. The values obtained seem to present themselves according to a definite pattern. After analysis of these values it appeared that equation 6,

$$\alpha = \frac{W}{W1s}\,{}^{3}\sqrt{K},$$

best fitted this relationship. For the dose of 8 mg. carbon/100 g. this equation becomes

$$\alpha = 6{\cdot}0 = \frac{W}{W1s}\,{}^{3}\sqrt{K},$$

α being the corrected granulopectic index, and after calculating the average for all the values of α for the dose of 8 mg. carbon/100 g. the curve describing this relationship was drawn in Fig. 9. It is apparent that the values of K with respect to $\frac{W}{W1s}$ follow more or less this relationship. The individual variations, once the correction is made, are very much smaller. In the Table the values for the granulopectic index K and for the corrected granulopectic index α calculated from a large number of animals are presented with their respective standard deviations.

TABLE.—*Granulopectic Index K and Corrected Granulopectic Index α with their Respective Standard Deviations.*

Dose of carbon (mg./100 g.).	No. of rats.	Granulopectic Index.	Corrected granulopectic index.
4	16	0·062 ± 0·029	8·5 ± 0·91
8	100	0·026 ± 0·014	6·0 ± 0·45
16	37	0·013 ± 0·004	4·5 ± 0·30
32	15	0·006 ± 0·0018	3·6 ± 0·37
48	10	0·0042 ± 0·0014	3·2 ± 0·39

The granulopectic activity of the cells of the R.E.S. seems to vary with the cube of the relative weight of the organs involved The reason for this relationship is not clear and will necessitate further investigations, but the knowledge of it allows us better to analyse and compare the data from different animals.

DISCUSSION.

When carbon particles are injected intravenously, the rate of clearance of carbon from the blood is governed by an exponential equation ; this same type of equation expresses the clearance of colloidal gold (Barrow, Tullis and Chambers, 1951 ; Sheppard, Gilbert and Hahn, 1951 ; Zilversmit, Boyd and Brucer, 1952), of colloidal zirconium and yttrium (Dobson, Gofman, Jones, Kelly and Walker, 1949), of colloidal iron saccharate (Nissim, 1953) and colloidal chromium phosphate (Gabrielli, 1951).

This seems to be the general way in which inert particulate matter is cleared from the blood. The reasons for this exponential relationship were not clear, especially since there is ample evidence that particles phagocyted by the R.E.S. diminish its further capacity for phagocytosis. This was demonstrated for carbon

particles (Halpern *et al.*,1950) and for colloidal iron (Gabrielli and Holmgreen, 1952). The exponential nature of the clearance of particles from the blood cannot, therefore, be explained on the basis of an analogy with the other clearance curves of diffusible material or substances excreted by the kidney.

In this study an effort was made to explain in the case of carbon particles the nature of the mechanisms which determine the rate of clearance of particles from the blood, introducing such notions as the inhibiting effects of the phagocyted particles on the granulopectic activity of the cells of the R.E.S.

An equation determining the rate of phagocytosis of carbon with respect to the amount of carbon injected and the amount of carbon phagocyted was evolved from a study involving the administration of various doses of carbon ; equation 5,

$$-\frac{d\,C}{d\,T} = 0{\cdot}06\left(1-\frac{P}{Co}\right),$$

expresses this activity. From this equation and from the values presented in Fig. 4 the simple factors governing the phagocytosis of particles can be ascertained. For all the doses of carbon injected the rate of phagocytosis of carbon particles always begins at a constant maximum speed which is independent of the concentration. However the rate of phagocytosis then decreases logarithmically showing the saturating effect on the cells. This decrease is relative, and depends on the ratio of injected carbon to that of phagocyted carbon. The saturating effect of particulate matter absorbed by the cells is partially offset by the stimulating effect of the particles in the blood. Starting phagocytosis at a given maximal speed, the cells of the R.E.S. decrease their activity much faster when small than when larger doses of carbon are injected. The exponential shape of the blood clearance curve is a reflection of the saturation of the cells by the absorbed particles. If the phagocyted particles did not saturate the cells, the curve of clearance of particles from the blood would continue as a straight line.

When the same doses of carbon are injected in different rats, the granulopectic index K of equation 1 expresses the difference in activity found in the animals. Some important knowledge can also be gathered from the calculation of the maximal initial speed of granulopexy for each animal from equation 1 to 5.

Gabrielli (1951) criticized methods using carbon particles on the ground that the phagocyted particles saturate the R.E.S. However, it follows from the data reported here that all colloidal suspension of particles whose concentration in the blood decreases according to an exponential equation must also saturate the R.E.S. Moreover, as the saturation of the R.E.S. is a relative factor, the objection presented by Gabrielli is not valid. The method using radioactive chromium phosphate proposed by this author presents no clear advantage over the one used in this study, with the added inconvenience that the amount of particulate matter injected is not always exactly known. Generally it can be said that any suspension of particles can be used to investigate the activity of the R.E.S. provided that it is homogeneous, stable and non-toxic. It would only be necessary to derive the equations expressing granulopectic activity for these particles in the same way as has been done for carbon particles.

The wide individual variations in granulopectic activity seen in different animals have been shown to be related to the weight of the organs, liver and spleen, principally concerned. The data presented in Fig. 9 and the Table suggest that

phagocytic activity varies with the cube of the weight of the liver and spleen. The explanation for this relationship is not yet clear although there is definite evidence, to be presented in another study, that the relative blood flow in these organs is an important factor. The corrections that can be made with the aid of this relationship greatly diminish the wide individual variations in granulopectic activity found in different animals. Care should be taken, however, of extraneous factors which could alter abnormally the weights of these organs (for instance factors causing extensive glycogen or fat deposition in the liver). In this case the corrected granulopectic index cannot be used.

The study presented here has been carried out with suspension of carbon particles of size of 250 to 500 Å ; the values presented are only valid for particles of this size. It is probable, as Dobson *et al.*, (1940) showed, that the larger the particles the faster the rate of clearance. If other particles than carbon are used, care should be taken also to have homogeneous and stable suspensions. The amount injected should also be quantitatively recovered in the organs which take up the particles. In the case of radioactive chromium phosphate Gabrielli (1952) recovered only about 50 to 55 per cent of the amount injected in the liver, spleen and lungs. This author injected also a constant amount of chromium phosphate without considering the weight of the rats, which varied form 80–300 g. He found that the rate of clearance of carbon from the blood varied but little in spite of it. On the basis of our experiments these results could be explained by the fact that small animals have a relatively larger liver and spleen. Therefore, if the small animals received relatively more carbon they had relatively larger organs to absorb them.

To confirm the results presented in this study, other experiments with other suspension of particles should be carried out to see whether these findings can be applied to all colloidal suspensions.

SUMMARY.

An attempt has been made to study quantitatively the factors which govern the granulopectic activity of the reticulo-endothelial cells of the spleen and liver in rats for carbon particles of about 250 Å. The most important factors which influence the speed of clearance of carbon from the blood are the quantity of carbon injected and the degree of saturation of the cells by phagocyted particles. An equation is proposed relating speed of phagocytosis to concentration of carbon in the blood and to the amount of carbon phagocyted. An activity index has been calculated for the rat.

Previous injections of carbon slow the rate of clearance of carbon for subsequent doses and the granulopectic index decreases considerably with consecutive doses, indicating a saturating effect.

By causing sudden modifications of the blood concentration of carbon during clearance experiments, it has been shown that the R.E.S. adapts itself rapidly to new conditions and changes its activity towards the particles according to the new level of carbon in the blood.

The granulopectic activity of the cells of the R.E.S. seems to vary with the cube of the relative weight of the organs, liver and spleen, principally involved. Although the reason for this relationship is not clear, it permits a better analysis of the data from different animals.

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THE ABSORPTION OF PARTICLES FROM THE PERITONEAL CAVITY IN RATS AND MICE TREATED WITH CORTISONE.

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SELYE (1949) stated that the movement of India ink from the subcutaneous tissues to the regional lymph nodes in the rat was accelerated by ACTH. Spain, Molomut and Haber (1950), using mice, found that the transport of India ink from the peritoneal cavity to retrosternal lymph nodes was retarded by cortisone. These reports are difficult to reconcile, and further investigation seemed justified.

Experiments were therefore carried out to determine the effect of cortisone on the rate of accumulation of radioactive glass microspheres in the retrosternal lymph nodes of rats and mice after intraperitoneal injection of these particles. Some similar experiments were also done with India ink.

MATERIALS AND METHODS.

The preparation and properties of radioactive glass microspheres, containing ^{134}caesium, have been described in a previous paper (Bangham, Magee and Osborn, 1953). In the samples used for the present experiments the size of the majority of the spheres lay between 0·5μ and 2μ. The suspension used for rats contained about 2000 and that for mice about 40,000 particles per ml., these concentrations having been found to be the lowest which would give convenient counting rates. The radioactivity was respectively *ca.* 1 μc. and 20 μc. per ml. Albino Wistar-strain rats (average weight 150 g.) and albino mice (average weight 23 g.) were injected intramuscularly with cortisone acetate suspension (Merck) for 3 days before the start of the experiments and the injections were continued until the animals were killed. The daily dose used was 5 mg. in rats and generally 2·5 mg. in mice. For some experiments 2·5 mg. cortisone was given to the mice twice daily.

In rats the abdomen was opened under ether anaesthesia and the suspension of microspheres introduced directly into the peritoneal cavity. The incision was closed with a purse-string suture and the rat recovered rapidly. Mice were given the more concentrated suspension by simple intraperitoneal injection without anaesthesia. The animals were allowed free movement in their cages for varying times until they were killed with ether and coal gas. In rats the retrosternal lymph nodes were dissected individually; in mice the mediastinal contents were removed *en bloc*. The abdominal cavities were then opened, films of peritoneal fluid were made and specimens of spleen, omentum, liver and adrenal were taken for histological examination. The lymph nodes from the rats and the media-

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stinal contents from the mice were placed on glass coverslips 2 cm. in diameter and dried in an oven at 95° overnight. After transfer to a muffle furnace they were gradually heated to 300°, kept at this temperature for 1 hr. and then ashed between 470° and 500° for about 10 hr. When cold, a drop of water was added to the ash on each coverslip and allowed to evaporate, thus fixing the ash firmly to the glass in a thin layer. The ashed specimens were assayed for radioactivity, using a G.E.C. end-window counter type E.H.M.2 and a Panax scaling unit. The technique of ashing differed from that described by Bangham *et al.* (1953), who found a marked fall in the radioactivity of specimens ashed on nickel planchettes at 600°. This appeared to be due to changes in the metal surface. The use of glass coverslips and a temperature not exceeding 500° greatly reduced this loss and gave constant results. When lymph nodes were added to known quantities of microspheres on cover-slips this method gave a recovery of between 90 and 95 per cent. of the original activity.

The India ink used was Gurr's India ink for injection. This preparation is stated to contain 4 per cent carbon particles (size not exceeding 2μ) suspended in distilled water without gum and with a small amount of chloroform added as preservative.

RESULTS

Experiments with rats

Sixteen cortisone-treated male rats and the same number of control animals each received 1·0 ml. of the suspension of microspheres intraperitoneally and were killed in groups of eight, 1, 5, 24 and 48 hr. later. The mediastinal lymph nodes were assayed for radioactivity. No significant difference was found between the mean values of the control and experimental groups at any of the time intervals (Fig.).

The cortisone-treated rats showed loss of body wt. (average for 8 animals, 15 g.), shrinkage of the thymus and fall in spleen wt. (mean of controls 1·08 g., mean of experimental, 0·81 g., difference 0·27, $p = 0·05$).

Experiments with mice

Sixteen cortisone-treated mice (6 male and 10 female) and a group of control animals corresponding in sex and weight were given 0·5 ml. of microsphere suspension intraperitoneally. Half the animals were killed 2 hr., and the remainder 24 hr. after the injection. The radioactivity of the mediastinal contents was measured, and again no significant difference was found between the mean values in the control and experimental groups at either time (Table).

The absorption of microspheres did not, then, show a retardation with cortisone similar to that reported by Spain *et al.* (1950); accordingly further experiments were undertaken, using India ink, in an attempt to reproduce their results.

A cortisone-treated group of 8 male and 3 female mice, and a corresponding group of untreated animals were injected intraperitoneally with 0·5 ml. undiluted India ink. Nine treated and 6 control mice died at varying times, and the remainder were killed, some at 16 hr., and some at 24 hr. after the injection. The retrosternal nodes of all the animals were jet black, and in many from each group the retrosternal and diaphragmatic lymphatic vessels were clearly visible as black lines.

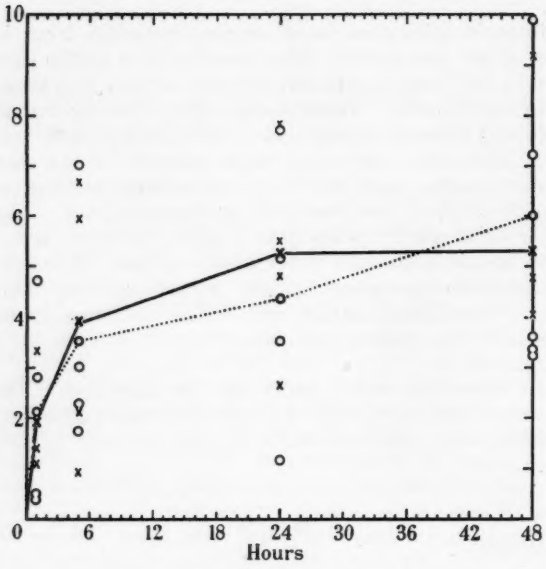

FIGURE.

Ordinate. Percentage of intraperitoneal dose of radioactive microspheres found in retrosternal lymph nodes.

Abscissa. Time in hours.
—x— Control rats. ...○... Cortisone-treated rats. Lines drawn through means.

TABLE.—*Radioactivity of Mediastinal Contents (Counts per Minute ÷ 32)*

	After 2 hr.	After 24 hr.
Control mice	13·5	0·5
	89·0	51·7
	104·3	19·5
	136·8	21·9
	31·6	85·6
	3·2	41·3
	290·5	5·6
	23·5	19·6
Means	86·55 (1·3 per cent injected dose)	30·71 (0·5 per cent injected dose)
Cortisone-treated mice	10·6	197·6
	34·2	1·0
	135·3	14·2
	16·4	58·4
	100·0	85·5
	22·3	11·7
	112·5	140·3
	92·7	155·8
Means	65·5 (1·0 per cent injected dose)	83·06 (1·3 per cent injected dose)
Difference between means—	Difference between means—	
= 21·05	=52·35	
$t = 0·5535$	$t = 1·1971$	
$P > 0·5$	$P > 0·2$	

The experiment was repeated with similar groups of mice using India ink diluted 1 : 10 with 0·9 per cent NaCl, cortisone administration being continued throughout. Two experimental and two control animals were killed and examined at intervals of 1, 3, 5 and 7 days after injection. The 3 remaining cortisone-treated mice died, 1 on the 12th and 2 on the 13th day. In a further experiment with 6 mice, twice the dose of cortisone was used, i.e., 2·5 mg. twice daily. Groups of these animals were injected with India ink on the 3rd, 5th and 6th days of cortisone treatment, and were killed 1 hr. after injection. In both these experiments the retrosternal lymph nodes were black in every mouse, those from the treated mice were smaller but otherwise showed no difference in appearance.

To determine whether cortisone had an effect on the early stage of absorption 3 experimental and 3 control animals were killed 15 min. after the injection of ink. In one animal from each group ink was not evident in the nodes, but in the remainder, either one or two nodes contained carbon particles.

The cortisone-treated mice showed changes at autopsy similar to those seen in the rat but of greater severity. The mean spleen weight of the experimental animals was 40 per cent of that of the controls ($p < 0{\cdot}02$). There was pallor of the liver, due to increased glycogen content, demonstrated in sections by Best's carmine stain.

DISCUSSION.

The properties of radioactive glass microspheres have been discussed by Bangham *et al.* (1953). In the present experiments material of greater radioactivity has been used, and consequently it has been possible to use suspensions containing fewer particles. Thus the number of particles in a single mediastinal lymph node of a rat 24 hours after injection is only of the order of 20,000, and in fact the glass spheres are difficult to find microscopically in sections. This, of course, is in marked contrast to the state of the blackened lymph nodes in animals injected with India ink, where the number of particles present is relatively enormous. As emphasised by Bangham *et al.* (1953) the draining lymph nodes are capable of accommodating much larger numbers of microspheres, and it is therefore reasonable to suppose that effects due to saturation do not occur in our experiments, and that the radioactivity of the lymph nodes at any time during the experiment is a measure of the amount of particulate matter which has been carried to them (Efskind, 1940). The shape of the curve (Fig.) in the first 5 hours is similar to that found by Courtice and Steinbeck (1951) for the absorption of homologous plasma from the peritoneum of the rat, though a much smaller proportion of the particulate matter is absorbed (*ca.* 5 per cent of the particles as against *ca.* 98 per cent of the plasma). The flattening of the curve after 5 hours may be due partly to the small amount of free fluid remaining and partly to the adhesion of the particles to peritoneal surfaces, chiefly those of the omentum. Simer (1944) has studied this adhesion when India ink is injected, and believes that most of the particles are at first extracellular, being possibly held by fibrin, and only later undergo phagocytosis. Our histological findings with India ink support this.

The dose of cortisone given to our rats was the same as that used by Magarey and Gough (1952) and Buck and Wilhelm (1952), who found this quantity sufficient to produce toxic changes after several days of continuous administration. The curves shown in the Figure suggest that the administraton of cortisone in this dosage was without effect on absorption of the particles. The differences

between the means of control and experimental groups are not significant at any of the time intervals.

The results with mice (Table) show a much greater scatter, both in control and experimental animals, than those with rats. To find out whether this might be due to errors of injection technique, an operative method similar to that employed with rats was used for 5 mice. There was no appreciable reduction in the scatter, and the problem requires further investigation. Statistical analysis of the Table does not reveal a significant difference between the mean values for the treated and untreated groups, but the scatter is such that an appreciable effect could be present without our being able to detect it.

Our results with India ink do not confirm those of Spain *et al.* (1950), since all our mice killed later than fifteen minutes after injection showed deeply blackened lymph nodes, whether treated with cortisone or not. We cannot explain this discrepancy satisfactorily. It is possibly due to a difference in the composition of the inks used. Florey (1927) found India ink unsuitable for experiments on peritoneal drainage because of its tendency to coagulate in the presence of body fluids, and this tendency may vary with different preparations. Another possible explanation is suggested by the fact that Spain *et al.* found a reduction in the mean spleen weight of cortisone-treated mice to 20 per cent of that of controls, whereas in our experiments the reduction was only to 40 per cent and correspondingly greater shrinkage of the lymphoid tissue in their animals might have made the retrosternal nodes inconspicuous. However, this does not seem likely, since in our group of 6 mice given twice the daily dose of cortisone the mean spleen weight was in fact reduced to 20 per cent of that of the controls but the blackened retrosternal lymph nodes were still easily found.

Spain *et al.* (1950) have interpreted their findings to indicate that " cortisone treatment in mice results in a definitely retarded macrophage response." However it would appear that the influence of phagocytosis on the accumulation of particulate matter in the retrosternal lymph nodes is complex ; the ingestion of particles by fixed or resting cells in the omentum will retard it ; the activity of phagocytes within the lymph nodes will increase it by arresting escape, while the effect of phagocytosis by freely mobile cells in the peritoneal fluid is uncertain. There is evidence that many forms of particle can enter the diaphragmatic lymphatics without first being ingested by phagocytes (Florey, 1927 ; Allen and Vogt, 1937 ; Simer, 1944).

Gell and Hinde (1953), working with peritoneal exudate taken from mice, found that treatment of the animals with cortisone had no significant effect on the phagocytosis of killed *Staphylococcus albus* by macrophages. Observations made on films from the peritoneal fluid of our animals are consistent with this result, but detailed counts of ingested microspheres were not made because of the difficulty in distinguishing between round glass particles and other transparent globules in the cytoplasm of macrophages.

SUMMARY.

Suspensions of radioactive glass microspheres were introduced into the peritoneal cavities of rats and mice, and the rate of drainage via the diaphragmatic lymphatics was measured by assay of the retrosternal lymph nodes after varying times. No significant difference in absorption rate was found in either species

after treatment with cortisone in doses sufficient to produce characteristic general changes in the animals.

In similar experiments using mice only, cortisone was not found to influence the absorption of India ink particles.

We should like to thank Professor G. R. Cameron, F.R.S., for his advice and continuous encouragement, Drs. P. G. H. Gell and Isobel T. Hinde for allowing us access to their results, Dr. A. W. Steinbeck for invaluable discussion, Mr. S. B. Osborn for his expert advice on radioactivity problems and Mr. Please for help with the statistics.

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THE AGGLUTINATION OF MOUSE LEUCOCYTES BY ISO-IMMUNE SERA.

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ALTHOUGH the existence of iso-immune reactions has been recognised for over fifty years, it is only comparatively recently that suitable methods have been evolved for their recognition with red cells; it is therefore hardly surprising that no such methods have been available for leucocytes.

Much has been learnt from red cell agglutination about the antigenic composition of some normal and malignant tissues in mice, and the position to date has been stated recently by Gorer and Mikulska (1953). During the course of their work it became apparent that certain antigens present in both liver and leukaemic cells might be poorly represented in or absent from the red cell. The present work was undertaken to extend their work by providing an alternative method to red cell agglutination, and a technique for the specific agglutination of mouse leucocytes by iso-immune serum has been developed.

The action of heterologous sera on leucocytes and other tissues has been studied both *in vivo* and *in vitro*. A comprehensive summary up to 1927 is given by Lindström, who carried out a series of investigations on hospital patients suffering from leukaemia in an effort to treat them with an anti-leucocytic serum. More recently similar studies have been made in guinea-pigs by Chew and Lawrence (1937) and in rats by Cruickshank (1941). *In vitro* studies include those by Niven (1929) and by Hueper and Russell (1932) using tissue culture, and on leucocytic suspensions by Nungester, Gordon and Collins (1950).

Iso-immune sera were studied by Lumsden, who demonstrated an effect on rat tumour cells grown in tissue culture (1931) and later haemagglutination (1938). Phelps (1937) stated that an effect was also shown on cultures of splenic cells, but Medawar (1948) has thrown some doubt on his conclusions.

The present author in attempting to provide a method for demonstrating antigens absent from the red cell also used tissue culture, employing a modification of Jacoby's (1944) method. Numerous iso-immune sera were applied to actively growing cultures of both adult and embryonic tissues. The potency of the sera was known to be high by haemagglutination tests. No clear-cut result was obtained. This was in keeping with Medawar's (1948) observations on skin treated with iso-immune sera. On the other hand, treatment with heterologous immune serum (rat anti-mouse) produced changes identical with those described by Niven and others.

The effect of the sera on leucocyte suspensions was then tried, and it was found that although no morphological changes were seen, agglutination occurred in some cases after incubation for one hour. This observation has been developed.

While there may be some doubt as to the precise role played by antibodies in certain iso-immune reactions it seems clear that we may have to use methods for their detection different from those used in heterologous systems. Thus rhesus antibodies certainly play some part in the disastrous haemolytic reactions *in vivo* but do not produce haemolysis at all readily *in vitro*, and have not yet been shown to fix complement. These difficulties may arise from some peculiarity in the iso-antibody or in the antigens involved; the latter are probably very different from those determining species specificity.

MATERIALS AND METHODS.

Mice of four pure line strains were used, Bagg Albino (Balb) C, C57 Black, A, and C_3H. The origins of the mice have been described by Gorer and Mikulska (1953). They have been maintained by brother-sister mating.

Antibodies.—The antibodies described in this paper were prepared against an ascites-producing lymphogenous leukaemia occurring in the C57 Black strain and described by Gorer (1950) as E.L. 4. This antigen was chosen as it is very convenient to work with, the ascitic fluid consisting almost entirely of leukaemic cells, and because antibody titres against this antigen have been considerably higher than those produced against normal tissues. The form of agglutination reaction obtained with antibody appears similar whether E.L. 4 or normal tissues from C57 Blacks (liver, spleen or whole blood) are used as the antigen and any differences in titre may be explained on a quantitative basis. It appears that the antibodies evoked against a C57 Black mammary tumour may differ in some respects, but these reactions are still being investigated.

Antibody production.—The strains Balb C, A and C_3H are resistant to E.L. 4. A subcutaneous inoculation of 10–50,000 cells was followed after an interval of about a month, during which a small tumour usually formed and regressed, by a second intra-peritoneal inoculation of up to 90 million cells. The animals were bled 10 days after the second inoculation. A third and final injection was often made a month later and the animals subsequently bled out from the heart. The antibody mentioned in Table I was produced against a typical mammary adenocarcinoma occurring spontaneously in the A strain which has recently undergone transformation into a sarcoma. It has been used to illustrate a type of non-specific blocking antibody removed by absorption with liver, a phenomenon shown by other sera but not in so dramatic a form.

Leucocyte suspensions.—Cells from peritoneal exudates were used for all except preliminary testing as it was found to be impracticable to obtain sufficient cells from buffy coat. Various methods of producing an exudate were tried, including the injection of olive oil, liquid paraffin, turpentine, talc, Tyrode's solution, saline, Old Tuberculin, streptokinase, *Staphylococcus albus* and killed suspensions of *Staphylococcus aureus*, heat-killed *Mycobacterium tuberculosis*, *Erysipelothrix monocytogenes*, *Mycobacterium smegmatis* and B.C.G. Of all these agents, the last two have proved the most satisfactory.

The most cellular exudate was obtained by injecting a suspension of heat-killed *Staph. aureus* in a solution of Streptokinase-streptodornase (Burroughs Wellcome) 1000 units/ml., the yield reaching a total of 90 million cells on occasions. A mixture of turpentine in olive oil also gave a very cellular exudate, but both these methods had the disadvantage of giving cell suspensions that were very prone to form clumps on standing. This disadvantage may be of less moment when working with heterologous serum, but the less avid iso-immune serum sometimes gives results that are not sufficiently clear cut, especially in absorption experiments. This tendency to clump may be minimised by treating the suspensions with streptokinase. Less irritant agents such as liquid paraffin or Tyrode's solution do not give a high enough yield in an animal as small as the mouse.

Although the more cellular exudates gave the highest titres with antisera, those produced by B.C.G. or *Myco. smegmatis* were sufficiently reactive to be usable; the yield of 15–25 million cells was adequate for most experiments and spontaneous agglutination rarely encountered.

Mice aged 3–9 months were given a single intraperitoneal injection of 25–50 million organisms suspended in saline or Dubos medium. After an interval of 7–10 days for B.C.G. and 2–6 days for *Myco. smegmatis*, the animals were killed with chloroform and bled out

from the heart. If the bleeding was omitted the exudate tended to be contaminated with red cells. The peritoneum was then opened and washing of the viscera performed with successive amounts of about 0·5 ml. citrate saline, using a smooth-tipped Pasteur pipette, until the washing fluid was clear. This usually required about 2 ml. of fluid. The total exudate contained some 12–25 million cells of which 80–95 per cent were lymphocytes, 2–15 per cent monocytes or macrophages and a few polymorphs, the total and differential count varying to some extent with the strain. In particular *Myco. smegmatis* tended to provoke more macrophages in C57 Black and C_3H. The bodies were formolised or burnt and all instruments boiled after working with B.C.G. although the organism appears to be non-pathogenic for animals (Topley and Wilson, 1946).

Animals given B.C.G. and killed before the fifth or after the twentieth day have given reduced yields of feebly reactive cells. Increasing the vitamin C content of the diet (Nungester and Ames, 1948) has not led to increased cellularity of peritoneal exudates in mice.

The exudate was centrifuged at slow speed for 2 minutes and the supernatant discarded. The cells were then suspended in saline to a concentration of about 20,000/cu.mm. and then strained through a small pledget of cotton wool moistened with saline and packed into a small filter funnel. The final concentration was then adjusted to 12–14,000/cu.mm. by adding further saline. The suspensions should be used on the day of collection only, for although Steinberg and Martin (1946) found that their leukaemic cells remained stable for up to 4 days it has been found that normal mouse leucocytes give variable results after 24 hours.

TABLE I.—*The Development of Blocking Antibodies and their Removal after Absorption with Liver.*

Serum—Balb C anti-A Mammary Tumour obtained 22.xii.52.

Date of test.	Cells.	Reciprocals of titres.	
		Unabsorbed.	After absorption with Balb C liver.
23.xii.52	A	128	128
	Balb C	0	0
	C57 Black	64	128
2.i.53	A	0	256
	Balb C	0	0
	C57 Black	0	128

Absorption of sera.—The use of perfused liver for absorption has been described by Gorer and Mikulska. A similar technique was used in these experiments, but usually without the use of dextran. The liver required rather more washing but as dextran appeared to give slight but inconstant enhancement it was thought best to omit it. A few absorptions have been performed in the same way using suspensions of leucocytes or leukaemic cells. The results were in good agreement with the liver and will not be described separately. Control absorptions have been performed with each serum.

The agglutination test.—Doubling dilutions of serum in saline were prepared in precipitin tubes, 0·025 ml. volumes being used throughout. A volume of cell suspensions was then added, the tube shaken and incubated for 1½ hours.

For reading, each tube was given a flick with the thumbnail to dislodge the cells from the bottom of the tube and to break up the loose clumps that sometimes formed in the bottom of the controls. A portion was transferred to a slide and the result read microscopically. The aggregates were more stable than those of red cells and usually consisted of about 10–20 cells. Exudates containing large numbers of macrophages gave results more difficult to read than those composed almost exclusively of lymphocytes.

The best conditions for agglutination agreed closely with those found by Steinberg and Martin with heterologous serum. The optimum salt concentration was 0·85 per cent NaCl; at concentrations above 1 per cent agglutination tended to take place spontaneously. pH appeared to exert a complex effect, the highest titres being obtained at pH 6·8 but the largest clumps at 6·0–6·2. The reaction proceeded to completion in less than one hour at 23° but attained the same titre although rather more slowly at 37°. Centrifugation accelerated the reaction but did not greatly influence the titre.

Many attempts have been made to potentiate the sera. Human, horse and guinea-pig serum, bovine serum, albumin, dextran (Crookes Intradex 10 per cent in glucose), sucrose, trypsin, pre-treatment with heterologous immune serum, centrifugation and other methods have been tried and although some enhancement was found it was not sufficient to offset the risk of agglutination in the controls.

Controls.—As a check on their stability, cell suspensions were incubated in saline alone. The specificity of the sera was established by setting up a series of dilutions and adding cells of the donor strain. As a check on possible non-specific absorption or alternatively of the presence of naturally occurring non-specific agglutinins in the liver, absorptions have been made using liver from the donor strain and cells from the donor strain have been added to serum dilutions after absorption.

RESULTS.

The majority of the results agree qualitatively with those obtained by Gorer and Mikulska with the same sera although the titres obtained are often very different. In some instances haemagglutination will detect traces of antibody where the leucocytes do not. On the other hand, some antigens are present in leucocytes that are lacking in the corresponding red cells and their relative proportions appear to differ. The titres for the leucocytes of individual mice showed greater variability than for red cells, but this was to be expected and with repetition the effects of these differences were minimised.

Gorer (1947) pointed out that some sera lost their ability to agglutinate red cells after storage in the cold and suggested that this was due to the appearance of blocking antibodies rather than to loss of antibody. Further evidence on this point has been obtained by Gorer and Mikulska, who found that sera which become inactive on storage at — 20° when tested in saline medium or in normal human serum, showed undiminished activity in the presence of dextran after as long as 8 months' storage. Similarly some stored sera showed diminished ability to agglutinate leucocytes while remaining potent for red cells in dextran. A possibly related feature is that in the earlier stages at least, the serum regains its activity after absorption with liver. This is clearly shown in Table I. Serum previously very potent is seen to be inactive until after absorption when it regains its original titre. Other sera have also shown some enhancement after absorption with liver even when fresh, and this is reflected in the larger size of the clumps as well as in the rise in titre. The nature of this enhancement is at present unknown, it occurs irregularly and to a much lesser extent with haemagglutination; it may be due to the removal of non-specific blocking agents in the sera. Supernatant fluid from minced liver does not appear to effect it and no non-specific effect is shown by control absorptions. Forty-two sera have been investigated and none has shown non-specific effects with the donors' cells. Some of the sera prepared against normal tissues have been of low titre and one against E.L. 4 was inactive.

Balb C anti-E.L. 4.—A typical result giving a comparison with haemagglutination is shown in Table II. A and C_3H liver removed the antibodies for both A and C_3H cells leaving the titre for the Blacks unchanged. C57 Black liver removed all the agglutinins. Absorption with Balb C liver did not affect the titre and Balb C cells were not agglutinated.

C_3H anti E.L. 4.—Only two sera have been available. The first was a low titre serum giving titres of 32–64 with C57 Blacks and 8 with A and Balb C. The second was a very potent serum giving titres of 256 with the Blacks and 32–64 with A and Balb C. Absorptions of the two sera gave similar results, but two absorptions were necessary with A and Balb C livers with the second serum.

Both A and Balb C removed the agglutinins for A and Balb C cells, leaving the titre for Blacks unaltered. Absorption with C57 Black liver removed the agglutinins. C_3H liver did not affect the titres and C_3H cells were not agglutinated (Table III).

TABLE II.—*Red and White Cell Agglutination by Serum Balb C Anti-E.L. 4 before and after Absorption with Liver.*

Cells.	Reciprocals of Titres.							
	Unabsorbed.		After absorption with A liver.		After absorption with Balb C liver.		After absorption with C57 Black liver.		After absorption with C_3H liver.	
	Red.	White.	Red.	White.	Red.	White.	Red.	White.	Red.	White.
A	16,000	64	0	0	16,000	64	0	0	128	0
Balb C	0	0	0	0	0	0	0	0	0	0
C57 Black	1,000	256	512	128	1,000	128	0	0	512	128
C_3H	4,000	64	0	0	4,000	64	0	0	0	0

The figures for white cells are from a typical single experiment; those for red cells are representative figures based on results made under varying conditions and are for comparison only.

TABLE III.—*Agglutinin Titres of Serum C_3H Anti-E.L. 4 after Absorption with Various Livers*

Single absorption with serum diluted 1/8.

Cells.	Unabsorbed.	Absorbed with liver from strain:			
		A.	Balb C.	C57 Bl.	C_3H.
A	64	0	0	0	128
Balb C	64	128	32	16	128
C57 Black	128	256	128	32	256
C_3H	0	0	0	0	0
After two Absorptions.					
A	64	0	0	0	64
Balb C	64	0	0	0	128
C57 Black	128	128	64	0	256

A anti-E.L. 4.—This has proved a difficult serum to prepare; some have had very low titres and reacted on the day of collection only and one was inactive, but sufficient data have been obtained from the 5 sera which have proved reasonably active. The most potent agglutinated Balb C cells to a titre of 64, C57 Black to 256 and C_3H to 64–128. Weaker sera agglutinated C_3H cells very feebly, and the weakest agglutinated only the C57 Black cells. Results of absorption of a strong serum are shown in Table IV. Absorption with Balb C liver removed the antibodies against Balb C and C_3H cells. C57 Black liver removed all the antibodies and C_3H reduced those for itself alone, leaving the titre for Balb C and

TABLE IV.—*Agglutinin Titres of Serum A Anti-E.L. 4 after Absorption with Various Livers.*

Two absorptions with serum diluted 1/16.

Cells.	Unabsorbed.	Absorbed with liver from strain:			
		A.	Balb C.	C57 Bl.	C_3H.
A	0	0	0	0	0
Balb C	64	64	0	0	64
C57 Black	128	256	512	0	512
C_3H	64	128	0	0	16

C57 Black unaffected. A liver had no effect on the titres and A cells were not agglutinated.

DISCUSSION.

Comparison between results obtained with red and white cells show very good agreement if allowance is made for differences in titre—which are in themselves immaterial—and the same antigens are seen to be present in the two types of cell. The proportions of antigen appear to be different and there is much less difference between the titres obtained with the leucocytes of the four strains than with red cells. With Balb C anti-E.L. 4 the red cell titre for A cells is up to 16,000, with C57 Black 1–2,000. The corresponding white cell titres are 64 and 128. With another serum not here described, the titre for both red and white cells was 128.

The antigens that can be enumerated from the above experiments are the same as those recently described by Gorer and Mikulska with two additions. A anti-E.L. 4 serum agglutinates only C57 Black red cells; on the other hand with the white cells it reacts with C57 Black, Balb C and C_3H. From the absorption experiments, these two further antigens appear to be independent. Other experiments have been performed on some six or more different types of serum and on the whole good agreement is given. It is hoped to complete these results in detail with an analysis of the antibodies produced against different types of tumour and normal tissues in a further paper.

The importance of this technique lies in its possible application to other iso-immune systems, in particular those where haemagglutination has no application. As Medawar (1946) has pointed out, although white cells may act as very potent antigens nothing is known of any possible antibodies formed against them after fresh blood transfusions. Although their practical importance is apparently slight it also appears from his work that there may be a close antigenic connection between skin and leucocytes and it is possible that an application may be found in the realm of skin grafting. Preliminary experiments show that after a single graft, antibodies may be detected. In this laboratory it is also proposed to use the technique to demonstrate differences between normal and malignant tissues and again preliminary results are encouraging.

It is emphasised that the preparation of the leucocyte suspension must be standardised. The agglutinability of leucocytes is more variable than that of erythrocytes and occasionally the cells from an individual mouse may fail to react with any serum. It would be desirable to set up a control titration with a known serum in certain cases.

SUMMARY.

Suspensions of mouse leucocytes suitable for agglutination may be obtained from the peritoneal exudate following the injection of *Myco. smegmatis* or B.C.G.

These have been studied principally in connection with sera produced against C57 Black leukaemia cells (E.L. 4) in three resistant strains, A, Balb C and C_3H.

With the sera produced by the two latter strains all results of agglutination and absorption agreed with those obtained by means of red cell agglutination.

With serum produced by the A strain the employment of white cells served to demonstrate two antibodies not shown by red cell agglutination.

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